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13. ABSTRACT (Maximum 200 Words)

The project is designed to evaluate anticonvulsant and neuroprotectant properties of novel medical countermeasures to lithium-pilocarpine (Li-pilo) induced status epilepticus (SE) used here as a model of organophosphorus nerve agents. The nonbarbiturate anesthetic propofol was found to induce significant anticonvulsant and neuroprotectant effects in Li-pilo SE. The strychnine-insensitive glycine site partial agonists 1-aminocyclopropanecarboxylic acid (ACPC) and D-cycloserine had no anticonvulsant activity but ACPC induced significant neuroprotection. Although ongoing, studies involving the spin-trap N-*tert*-α-(2 sulfophenyl) nitrone (S-PBN) and the nonsteroidal anti-inflammatory drug mefenamic acid indicate neuroprotection without anticonvulsant activity for both agents. It is concluded that neuroprotection may be induced even in intractable seizures.

14. SUBJECT TERMS
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INTRODUCTION

The project tests novel treatment strategies for the N-methyl-D-aspartic acid (NMDA) component of organophosphorous (OP) nerve agents that are intended to complement or improve current treatments. OP agents induce an initial cholinergic over stimulation that is followed by a glutamatergic over stimulation of NMDA receptors. The excessive NMDA activation and excitotoxicity results in status epilepticus (SE), neurological deficit and neuropathology associated with OP intoxication. The lithium-pilocarpine (Li-pilo) model of cholinergic convulsions in rats is used as the experimental model of OP nerve agent-induced SE. The project tests compounds that act as anticonvulsants in that they inhibit the seizures by acting on the strychnine-insensitive glycine site of the NMDA receptor. The project also tests neuroprotectants that protect the brain from seizure-induced neuropathology by interfering with NMDA receptor-mediated intracellular excitotoxicity mechanisms. Experimental parameters used in these studies to assess the test drug activity are: 1) electrocorticograph (ECoG) determination of continuous high amplitude spiking as a measure of SE duration; 2) spontaneous activity before and after SE as a measure of neurological deficit; 3) neuropathology in brain regions damaged by Li-pilo SE.

During the past year scheduled experiments testing 1-aminocyclopropranecarboxylic (ACPC) and D-cycloserine (DCS) have been completed. Those compounds were tested because they are partial agonists of the strychnine-insensitive glycine site on the NMDA receptor ionophore complex (Watson et al., 1990; Watson and Lanthorn, 1990). It was hypothesized that those compounds would inhibit the NMDA receptor component of the Li-pilo SE (Ormandy et al., 1989; Walton and Treiman, 1991) and act as anticonvulsants in cholinergic convulsions. The results of those experiemtns have been prepared in manuscript form and submitted to

NeuroToxicology. The outcomes of those studies are discussed in this report and the manuscript is included in the Appendix.

α-phenyl-N-tert-butylnitrone (PBN) is a spin-trapping agent meaning that it reacts with reactive oxygen species (ROS) to form a more stable nitroxide free radical (Floyd, 1990). When tested directly in brain injury models, PBN has been shown to reduce hydroxyl radical production (Sen et al., 1994; Marklund et al., 2001a). Such an action would be neuroprotective against NMDA mediated toxicity which is hypothesized to be mediated by ROS mechanisms (Bruce and Baudry, 1995; Rong and Baudry, 1996; Rong et al., 1999).

PBN is neuroprotective in experimental models of NMDA-induced neurotoxicity in rat cerebellar granule cell cultures (Lafon-Cazal et al., 1993a; Lafon-Cazal et al., 1993b; Yue et al., 1992). PBN also reduces the forebrain damage found in CNS ischemia models in gerbils (Yue et al., 1992; Oliver et al., 1990; Hall et al., 1993) and in rats (Cao and Phillis, 1994; Zhao et al., 1994; Folbergrova et al., 1995; Li et al., 2001), even when administered as late as 12 hours after the ischemic episode (Cao and Phillis, 1994). PBN has been shown to be neuroprotective in experimental models of epilepsy. 200 mg/kg PBN administered 30 minutes before kainic acid had no effect on the SE but induced a neuroprotective effect as measured by cytochrome C oxidase activity and energy metabolism (Milatovic et al., 2001). 100 mg/kg PBN was neuroprotective in flurothyl-induced SE (He et al., 1997).

N-*tert*-butyl-α-(2 sulfophenyl) nitrone (S-PBN) is a sulfonated derivative of S-PBN that is significantly more water-soluble. Like PBN, S-PBN is a spin-trapping agent. As described below, S-PBN is at least as active a neuroprotectant as PBN when tested *in vivo*. The major difference between PBN and S-PBN is pharmacokinetic. PBN has a plasma half-life of 3 hours and readily penetrates the blood brain barrier (BBB) (Chen et al., 1990). S-PBN is reported to have a plasma half-life of 9 minutes and poor BBB penetration (Marklund et al., 2001a; Yang et al., 2000). Despite these reports systemically administered S-PBN induces significant CNS neuroprotection when tested using *in vivo* models of neural injury. For example, systemically administered S-PBN decreased central lesions induced by numerous excitotoxins including NMDA, AMPA, kainic acid, 3-acetylpyridine, MPP^+ and malonate (Schulz et al., 1995a). Systemic S-PBN reduced hypoxic lesion volume when tested as late as 6 hours following the ischemic episode (Schulz et al., 1995b). Intraperitoneal PBN or S-PBN were equally effective in reducing infarct volume following embolic stroke in rats (Yang et al., 2000). Intravenous PBN or S-PBN were equally effective neuroprotectants in traumatic brain injury (TBI) models in rats (Marklund et al., 2001a; 2001b; 2002). In two of the TBI studies S-PBN induced significantly greater neuroprotective effects than PBN (Marklund 2001a; 2002). Intraperitoneal S-PBN reduced the substantia nigra damage induced by MPP^+ in a rat model of Parkinson's Disease (Fallon et al., 1997). In spite of reportedly unfavorable pharmacokinetic properties, S-PBN induces significant neuroprotection in a wide variety of experimental models of excitotoxicity.

S-PBN was chosen to be tested in the project because of several advantages over PBN. As described above, systemically administered S-PBN was an effective neuroprotectant in numerous models of excitotoxicity. S-PBN was at least as effective as PBN and in several cases had

greater activity as a neuroprotectant. In addition, the aqueous solubility of S-PBN allowed administration of large doses in a smaller injection volume (4 mls/kg) than PBN.

BODY

Tasks Completed from the Approved Statement of Work

The project objective over the last 6 months was to use the Li-pilo model of SE in rats to test the anticonvulsant and neuroprotectant activity of N-*tert*-butyl-α-(2 sulfophenyl) nitrone (S-PBN) against the NMDA component of cholinergic convulsions. Li administration (3 mmol/kg, SQ) followed 20-24 hours later by pilocarpine (25 mg/kg, SQ) induces a SE of 2-3 hours duration that serves as a model of the convulsions induced by OP nerve agents. SE-induced afterdischarge was defined as the duration of continuous high amplitude ECoG spiking. Propofol (55 mg/kg, i.p.) was administered 3 hours following SE onset. As reported in the first progress report propofol significantly enhanced 24 hour survival without affecting the neuropathology induced by 3 hours of SE. The test compounds were to be administered either immediately following (within 1 minute) the pilocarpine administration (exposure treatment) or 5 minutes following the onset of SE (SE treatment). The objectives include testing saline as the vehicle control treatment for both the exposure and SE treatments. This was to be followed by a dose-response study of S-PBN, a novel neuroprotectant (Schulz et al., 1995a; 1995b; Marklund et al., 2001a; 2001b; 2002; Fallon et al., 1997).

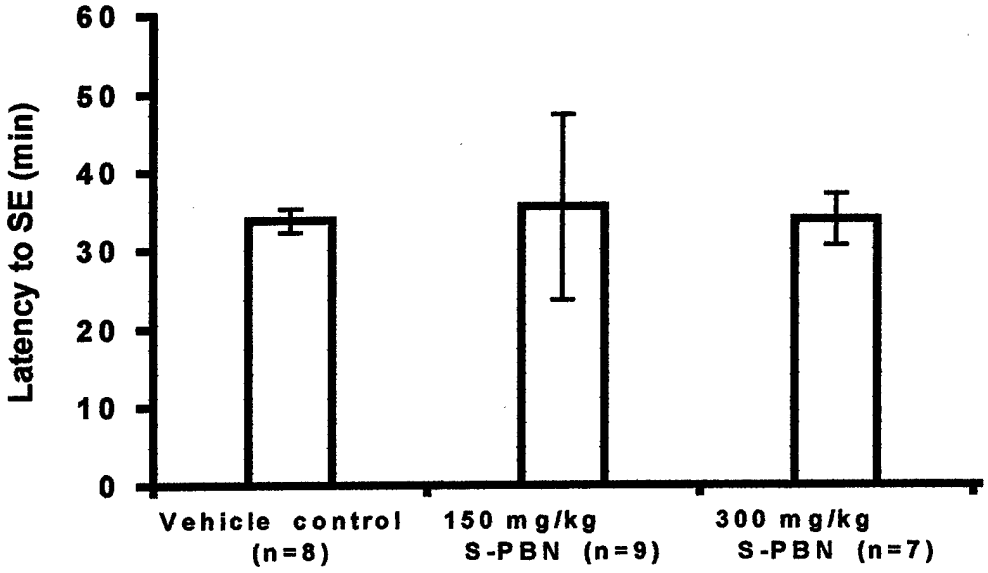


Figure 1. Latency to SE onset was the time from pilocarpine administration to the start of SE as defined by continuous high amplitude ECoG spiking. S-PBN administered immediately following pilocarpine (exposure treatment) had no significant effect on SE latency (one way ANOVA).

S-PBN had no anticonvulsant activity in Li-pilo-induced convulsions. The 150 and 300 mg/kg doses administered immediately following pilocarpine (exposure treatment) had no effect on the latency to SE onset (Figure 1). In addition, S-PBN had no significant effect on SE afterdischarge duration whether administered either as exposure treatment or following 5 min of SE (Figure 2).

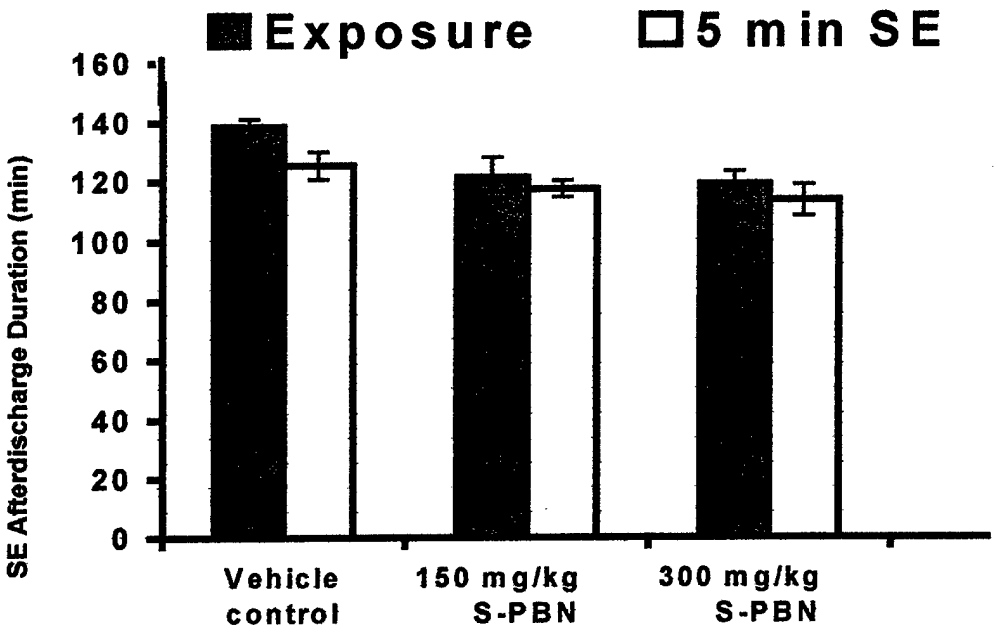


Figure 2. SE afterdischarge duration was defined as the period of continuous high amplitude ECoG spiking. Administration of 150 or 300 mg/kg S-PBN either immediately following pilocarpine (exposure) or after 5 minutes of SE had no significant effect on SE afterdischarge duration (one way ANOVA).

Spontaneous activity was monitored as a measure of the neurological deficit induced by SE and the possible neuroprotection that might be induced by S-PBN. Spontaneous activity was monitored for 10 minutes in an activity monitor the day of the lithium administration (preseizure) and again 24 hours following pilocarpine administration. In vehicle control animals, the Li-pilo SE produced a decrease in distance traveled (DT) and an increase in resting time (RT) indicating a neurological deficit (Figures 3 and 4) (Lallement et al., 1997; Lallement et al., 1998; Walton and Treiman, 1991). S-PBN treatment either at exposure or following 5 minutes SE had no statistically significant effect on the SE-induced neurological deficit (one way ANOVA) (Figures 3 and 4).

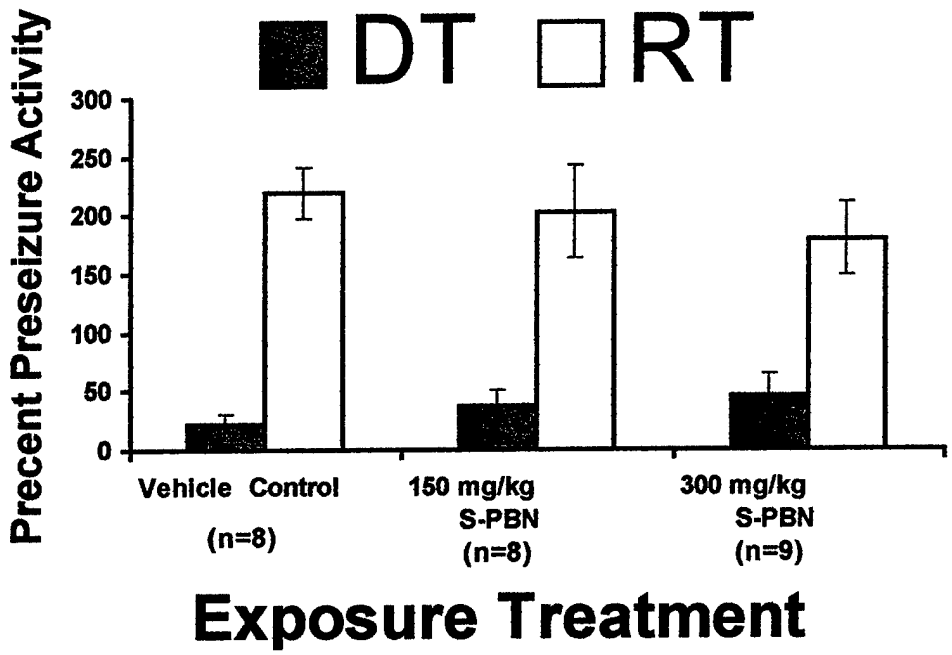


Figure 3. Effects of Li-pilo SE on spontaneous activity expressed as a percent of preseizure activity. Spontaneous activity was determined for 10 minutes in an activity monitor. Distance traveled (DT) was decreased and resting time (RT) increased indicating a SE-induced decrease in spontaneous activity. S-PBN exposure treatment had no effect on the neurological deficit.

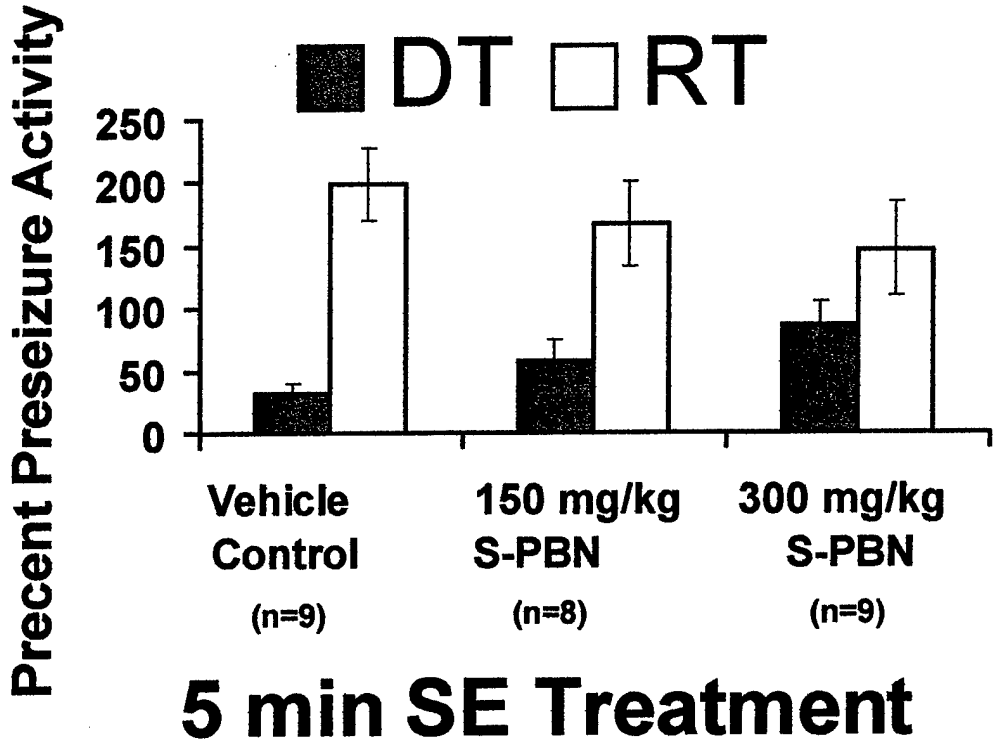
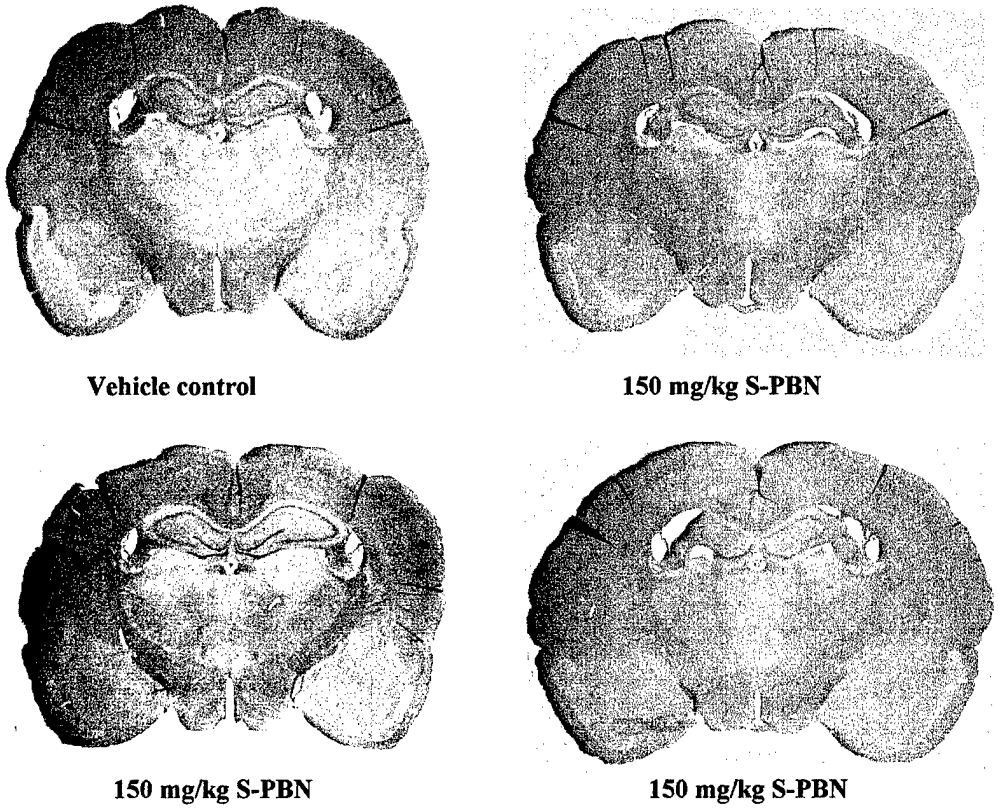


Figure 4. S-PBN administration following 5 minutes of Li-pilo-induced SE (SE treatment) also had no effect on neurological deficit as measured by distance traveled (DT) and resting time (RT).

Twenty-four hours following pilocarpine administration and immediately following spontaneous activity assessment the animals were sacrificed and perfusion fixed for histological anaylsis of SE-induce neuronal damage. The 24 hour survival period is optimal for the demonstration of neuropathological damage in both Li-pilo (Clifford et al., 1987; Fujikawa, 1996) and soman SE (McDonough et al., 1998). Formalin-fixed brains were removed and embedded in paraffin for sectioning. A 5 μM section was taken every 150 μM through the brain tissue 0.8 to 4.8 mm posterior to bregma. This area was chosen for the histopathological analysis for efficiency and because it contains critical brain nuclei that exhibit the greatest degree of damage from soman (McDonough et al., 1998) and Li-pilo convulsions (Clifford et al., 1987; Motte et al., 1998; Fujikawa et al., 1999; Peredery et al., 2000). The sections were prepared with hematoxylin and eosin (H&E) staining and sent to the coinvestigator, Dr. James Griffith of the Hershey Medical Center-Penn State University, for pathological analysis. While in progress, the analysis of the S-PBN sections was not completed in time for this biannual report.

Although the blinded histopathological analysis has not been performed, observation of whole H&E stained sections reveals markedly reduced neuropathology in the piriform and perirhinal cortex (Figure 5). S-PBN (150 mg/kg) administered as exposure treatment reduced the macroscopic lesions typically observed in the piriform and perirhinal cortices. Although preliminary, this data suggests S-PBN induces neuroprotection without anticonvulsant activity. It has been proposed that because of acetylcholinesterase inhibitor activity PBN is anticonvulsant in diisopropylphosphorofluoridate or physostigmine SE but inactive in SE induced by cholinergic agonists (Zivin et al., 1999; Milatovic et al., 2000). Our preliminary data supports the evidence that S-PBN has no anticonvulsant activity in cholinergic agonist convulsions but suggest that S-PBN is neuroprotective in Li-pilo-induced SE.



Vehicle control **150 mg/kg S-PBN**

150 mg/kg S-PBN **150 mg/kg S-PBN**

Figure 5. Representative H&E sections of rat brains demonstrating the extent of Li-pilo-induced neuropathology and the neuroprotection induced by S-PBN. The macroscopic lesions in piriform and perirhinal cortex appears to be greatly reduced following S-PBN exposure treatment. S-PBN did not affect the seizure activity.

Studies with mefenamic acid have been initiated as scheduled. As indicated in the original proposal, 40 mg/kg mefenamic acid administered as exposure treatment prevents the onset of Li-pilo SE. Of the 6 animals tested to this point, 3 failed to develop SE. As indicated in Figure 6, no macroscopic lesions were observed in the temporal lobe regions of the rats without SE. The remaining 3 rats treated with 40 mg/kg mefenamic acid as exposure treatment experienced the expected Li-pilo SE but appeared to have reduced brain damage as determined by observation of the apparent macroscopic lesion volume.

Mefenamic acid is a nonsteroidal anti-inflammatory drug (NSAID) that induces anticonvulsant activity in experimental models of epilepsy. Forty mg/kg mefenamic acid completely inhibits the seizures and neural damage induced by a convulsive dose (380 mg/kg) of pilocarpine (Ikonomidon-Turski et al., 1988). Mefenamic acid is also an effective anticonvulsant in focal and systemic penicillin seizures (Wallenstein, 1991; Wallenstein and Mauss, 1984; Wallenstein, 1987). The initial experiments of this project have also found anticonvulsant activity although the seizure inhibition is not as total as reported for pilocarpine seizures (Ikonomidou-Turski et al., 1988).

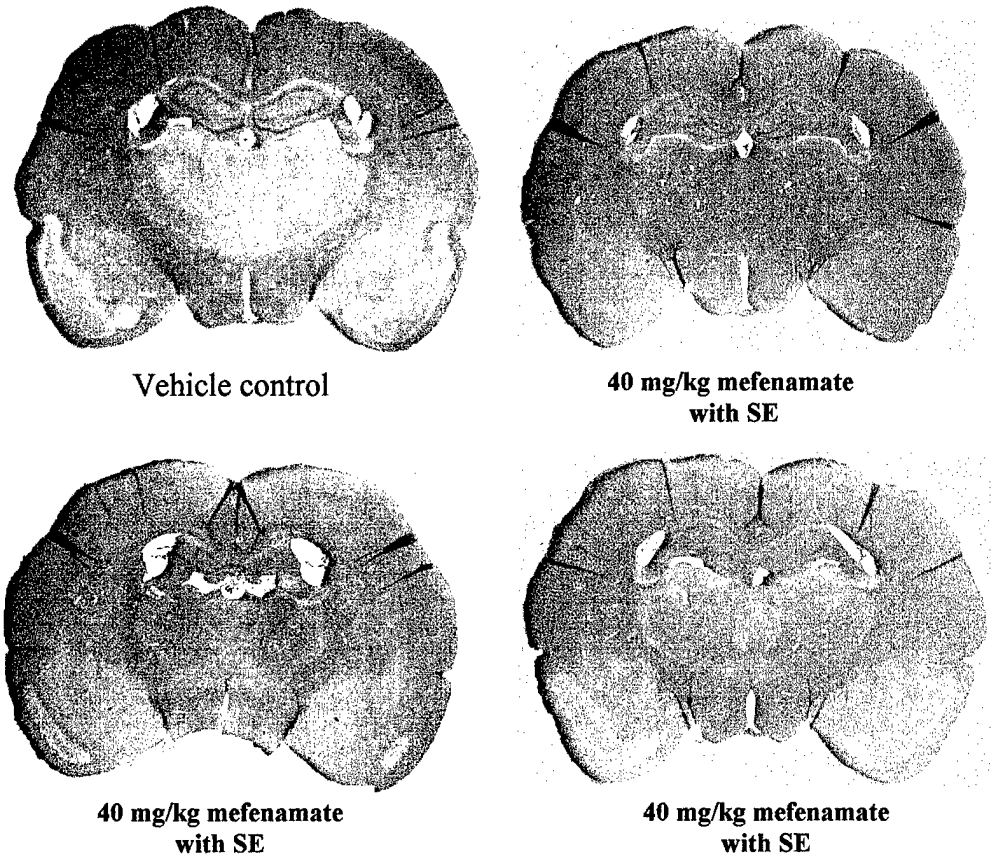


Figure 6. Representative H&E sections of rat brains demonstrating the extent of Li-pilo-induced neuropathology and the neuroprotection induced by mefenamic acid. Mefenamic acid (40 mg/kg, sc) was administered as exposure treatment. In one case (upper right) no SE was produced and no apparent neuronal damage occurred. In the case of the bottom 2 sections, SE was induced but the macroscopic lesions appear less severe than that in the vehicle control animal.

Even when Li-pilo SE is not prevented, mefenamic acid appears to reduce the macroscopic lesion size in piriform and perirhinal cortex (Figure 5). Although the study is ongoing and these results do not include histological evaluation of H&E stained sections, these data indicate mefenamic acid may be neuroprotective. If so, mefenamic acid would be similar to ACPC and possibly S-PBN by inducing neuroprotection activity without inhibiting ongoing SE. Mefenamic acid has been reported to be a competitive NMDA receptor antagonist which would induce neuroprotection by reducing NMDA receptor mediated excitotoxic insult (Chen et al., 1998).

As indicated in Reportable Outcomes, 2 manuscripts have been submitted for publication during the reporting period. The manuscripts are attached in the Appendix. The major findings of those manuscripts are included below and in the section "Completed Tasks Outside of the Approved Statement of Work"

Peterson, S.L., Purvis, R.S. and Griffith, J.W. Differential Neuroprotective Effects of the NMDA Receptor-Associated Glycine Site Partial Agonists 1-Aminocyclopropanecarboxylic Acid (ACPC) and D-Cycloserine in Lithium-Pilocarpine Status Epilepticus. Submitted to NeuroToxicology.

1. 1-Aminocyclopropanecarboxylic (ACPC) induced significant neuroprotection without affecting ongoing Li-pilo SE. Given the relatively short duration of action of ACPC, we hypothesize that additional doses would enhance the neuroprotection.
2. D-Cycloserine (DCS) had no effect on Li-pilo SE or the related brain damage. Given that DCS and ACPC are both partial agonists of the glycine site on the NMDA receptor, this data would suggest that partial agonist activity at that receptor is neither anticonvulsant nor neuroprotective.
3. ACPC neuroprotection is hypothesized to result from glutamate antagonist activity at NMDA receptors. Regional differences in ACPC neuroprotection is likely to result in regional differences in NMDA receptor subtype distribution as ACPC has variable affinities for the subtypes.

Completed Tasks Outside of the Approved Statement of Work

Peterson, S.L., Purvis, R.S. and Griffith, J.W. Anticonvulsant and Neuroprotective Effects of Propofol in Lithium-Pilocarpine Status Epilepticus. Submitted to Epilepsy Research.

1. Propofol induces significant anticonvulsant activity in Li-pilo SE. This is of interest because long lasting Li-pilo convulsions are more difficult to treat (Jope et al., 1986; Morriset et al., 1987; Walton and Treiman, 1988; Jones et al., 2002) and $GABA_A$ agonists lose potency as Li-pilo SE progresses (Walton and Treiman, 1988; Jones et al., 2002).
2. Multiple doses of propofol induce significant neuroprotection in Li-pilo SE. Propofol's NMDA antagonist (Orser et al., 1995; Ahmad and Pleury, 1995) and antioxidant properties (Murphy et al., 1992; 1993; Tsuchiya et al., 2001) provide neuroprotectant effects not available with other $GABA_A$ agonists employed in SE.

KEY RESEARCH ACCOMPLISHMENTS

- Collected all data for S-PBN study. Histopathological analysis of tissue is ongoing.
- Initiated study of mefenamic acid.
- Submitted 2 manuscripts for publication.

REPORTABLE OUTCOMES

- Peterson, S.L., Purvis, R.S. and Griffith, J.W. Anticonvulsant and Neuroprotective Effects of Propofol in Lithium-Pilocarpine Status Epilepticus. Submitted to Epilepsy Research.
- Peterson, S.L., Purvis, R.S. and Griffith, J.W. Differential Neuroprotective Effects of the NMDA Receptor-Associated Glycine Site Partial Agonists 1-Aminocyclopropanecarboxylic Acid (ACPC) and D-Cycloserine in Lithium-Pilocarpine Status Epilepticus. Submitted to NeuroToxicology.
- Peterson, S.L., Purvis, R.S. and Griffith, J.W., Propofol Inhibition of Lithium-Pilocarpine-Induced Status Epilepticus. Epilepsia 42 (S7): 19 (2002). Abstract.

CONCLUSIONS

The project is on schedule. There are no anticipated complications that will prevent completion of all approved Statement of Work tasks as originally scheduled.

As part of this ongoing project we have found that the nonbarbiturate anesthetic propofol produces significant anticonvulsant and neuroprotectant activity in Li-pilo SE. Propofol totally suppresses all behavioral and electrographic seizure activity even after 3 hours of continuous SE. Additional doses of propofol also have a neuroprotective effect by reducing the size of the macroscopic malacic brain lesions by 90%.

Propofol's anticonvulsant activity is mediated by both $GABA_A$ agonist activity and NMDA antagonist activity (Orser et al., 1995; Ahmad and Pleuvry,1995). Propofol is anticonvulsant in various experimental models of epilepsy (Rasmussen et al., 1996; Lowson et al., 1990; Lee et al., 1998; Holtkamp et al., 2001) and human SE (Kuisma and Roine, 1995; Stecker et al., 1998; Prasad et al., 2001). Propofol is also an antioxidant that acts as a free radical scavenger (Murphy et al., 1992; Murphy et al., 1993; Tsuchiya et al., 2001). This is a critical difference from other $GABA_A$ agonists given the predominate role of glutamate and NMDA receptors in both Li-pilo and organophosphorus nerve agent mediated SE (Ormandy et al., 1989; Walton and Treiman, 1991; McDonough and Shih, 1993; McDonough and Shih, 1997). It is hypothesized that the SE-induced NMDA receptor activation leads to an increased production of reactive oxygen species (Lafon-Cazal et al., 1993a; Coyle and Puttfarcken, 1993; Michaelis, 1998) that mediate the SE neuropathology (Bruce and Baudry, 1995; Rong and Baudry, 1996; Rong et al., 1999; Peterson et al., 2002). We propose that propofol's NMDA antagonism would attenuate the NMDA receptor–induced excitotoxicity while the antioxidant activity suppresses neuronal destruction by the reactive oxygen species. In support of this hypothesis, propofol has been shown to be anticonvulsant against NMDA-induced convulsions in mice (Ahmad and Pleuvry, 1995) and to

be neuroprotective in ischemic stroke by a proposed antioxidant mechanism (Young et al., 1997; Gelb et al., 2002; Wang et al., 2002). Our own studies demonstrate remarkable anticonvulsant and neuroprotectant activity in cholinergic convulsions. In addition, propofol is commercially available and FDA approved which would facilitate immediate deployment.

Our initial studies have shown that ACPC is neuroprotective without inducing anticonvulsant activity. While still in progress, our studies also indicate that S-PBN and mefenamic acid are neuroprotective without being anticonvulsant. This raises the interesting possibility that brain damage may be reduced even during intractable seizures.

Evidence from other laboratories supports our contention that neuroprotection does not require the elimination of seizure EEG afterdischarge. For example, competitive and noncompetitive NMDA antagonists (ketamine, MK-801, CGP 40116) induce only partial inhibition of Li-pilo-induced EEG afterdischarge but induce significant reductions in neuropathology (Ormandy et al., 1989; Walton and Treiman 1991; Fujikawa et al., 1994 and 1995). In pilocarpine SE the neuropathology is inhibited by NMDA receptor antagonism in spite of unabated EEG activity (Rice and DeLorenzo, 1998). Our own results indicate that ACPC reduces neuronal damage by competitive NMDA antagonism without affecting seizure afterdischarge.

Studies with other drugs have also shown neuroprotection in Li-pilo SE. Vigabatrin administered 10 minutes following pilocarpine administration and then daily for 45 days after SE reduced damage in the hippocampus but exacerbated damage in the entorhinal cortex (Andre et al., 2001). Pregabalin administered 20 minutes following pilocarpine and daily for up to 50 days after SE reduced damage in the piriform and entorhinal cortices but not the hippocampus (Andre et al., 2003). Caffeine administered 15 days before and 7 days after Li-pilo SE reduced damage in the hippocampus but exacerbated damage in the piriform cortex (Rigoulot et al., 2003). None of these treatments reduced the seizure activity which supports the hypothesis that neuroprotection is independent of anticonvulsant activity. However, each of those studies required chronic drug treatment and in the case of caffeine, pretreatment was also used (Rigoulot et al., 2003). In contrast, the neuroprotection observed in our studies by ACPC, S-PBN and mefenamic acid involved a single administration within the first hour following pilocarpine exposure. This suggests that drug treatment in the first few hours of SE is most critical. We also propose that repeated administration of ACPC, S-PBN or mefenamic acid during the first 4-6 hours of SE would provide even greater neuroprotection. Our own evidence that multiple propofol administration immediately following SE markedly enhances neuroprotection supports this hypothesis.

Given the evidence that neuroprotection does not require anticonvulsant activity, we hypothesize that neuronal damage and seizure activity involve related but independent mechanisms. Inhibition of SE is neuroprotective but neuroprotection does not require inhibition of SE. Because of this we are designing studies to develop drug treatment strategies that will potentiate or maximize neuroprotection during intractable seizures.

Neuroprotection implies that the brain has been protected from seizure-induced neuronal damage. Several drugs have been shown to protect the neuronal integrity and overall morphology of the brain following cholinergic convulsions (Fujikawa et al., 94 and 95, Eshhar et al., 1995, Andre et al., 2001, Rigoulot et al., 2003). However, the ultimate concern is whether the function of the brain has been protected by these treatments. Recently, Loscher's lab has reported that preservation of neuronal integrity and morphology in kainic acid SE does not prevent the eventual development of spontaneous seizures (Ebert et al., 2002, Brandt et al., 2003). This may be a significant concern as most, if not all, studies use neuronal integrity and morphology as the endpoints for successful treatment of status epilepticus (Dudek and Williams, 2003). It may be that "morphological neuroprotection" does not guarantee "functional neuroprotection" and that both must be assessed when evaluating pharmacological treatment of cholinergic nerve agents. Chronic treatment with pregabalin delayed (but did not prevent) the onset of Li-pilo SE-induced spontaneous recurrent seizures (Andre et al., 2003). This suggests functional neuroprotection is possible although the chronic pregabalin administration during the latent period complicates interpretation. Future studies of neuroprotection must be designed to assess components of brain function beyond the preservation of brain morphology.

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APPENDICES

1. Peterson, S.L., Purvis, R.S. and Griffith, J.W. Anticonvulsant and Neuroprotective Effects of Propofol in Lithium-Pilocarpine Status Epilepticus. Submitted to Epilepsy Research.

2. Peterson, S.L., Purvis, R.S. and Griffith, J.W. Differential Neuroprotective Effects of the NMDA Receptor-Associated Glycine Site Partial Agonists 1-Aminocyclopropanecarboxylic Acid (ACPC) and D-Cycloserine in Lithium-Pilocarpine Status Epilepticus. Submitted to NeuroToxicology.

Anticonvulsant and Neuroprotective Effects of Propofol in Lithium-Pilocarpine Status Epilepticus

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Abstract

Propofol is a nonbarbiturate anesthetic with anticonvulsant activity. In addition to being a $GABA_A$ agonist, propofol has NMDA antagonist and antioxidant properties, both of which would be a potential benefit in the prevention of status epilepticus (SE)-induced neuropathology. The anticonvulsant and neuroprotectant activity of propofol was tested in the lithium-pilocarpine (Li-pilo) model of SE in rats. When administered immediately following pilocarpine exposure, propofol (55 mg/kg, i.p.) prevented the onset of all motor and electrographic SE activity. When administered after 5 minutes or 3 hours of Li-pilo-induced SE, propofol inhibited all motor and electrographic seizure activity in addition to reducing mortality such that all rats survived the 24 hour period following pilocarpine administration. Rats experiencing 3 hours of SE prior to propofol administration demonstrated significant macroscopic and histologic lesions in the piriform and perirhinal cortices. However, rats administered an additional 20 mg/kg (i.p.) two hours following the initial 55 mg/kg propofol dose exhibited significantly less neuropathology as quantified by necrosis scores, optical density and lesion volume analysis. The neuropathology in the propofol extended treatment animals (55 mg/kg plus 20 mg/kg) was not significantly different from seizure control rats. The anticonvulsant and neuroprotectant benefit of the propofol extended treatment may result from NMDA antagonist and antioxidant activity. Because Li-pilo SE shares many characteristics of the treatment resistant cholinergic convulsions induced by organophosphorus nerve agents, the results of this study suggest propofol may serve as an effective treatment for nerve agent exposure.

1. Introduction

Pretreatment of rats with lithium chloride 20-24 hours prior to pilocarpine administration produces status epilepticus (SE) of several hours duration (Honchar et al., 1983; Clifford et al., 1987). If allowed to proceed the SE produces well-characterized lesions in the cortex, thalamus, hippocampus and amygdala regions (Honchar et al., 1983; Clifford et al., 1987; Motte et al., 1998; Peredery et al., 2000; Dube et al., 2000; Dube et al., 2001). Following a latent period of several weeks the animals that experience SE-induced neuropathology develop spontaneous limbic seizures (Lemos and Cavalheiro, 1995; Dube et al., 2000; Dube et al., 2001; Glien et al., 2001).

Lithium-pilocarpine (Li-pilo) convulsions model the cholinergic convulsions induced by the organophosphorus nerve agent soman. Both Li-pilo (Jobe et al., 1986; Morrisett et al., 1987) and soman convulsions (McDonough and Shih, 1993; Shih et al., 1997; McDonough and Shih, 1997) have an initial cholinergic phase that is inhibited by muscarinic antagonists. Both Li-pilo (Ormandy et al., 1989; Walton and Treiman, 1991) and soman convulsions (Shih, 1990; McLean et al., 1992; McDonough and Shih, 1993; McDonough and Shih, 1997) have a secondary glutamatergic phase that is inhibited by glutamate antagonists (McDonough and Shih, 1997; Solberg and Belkin, 1997). Both produce a similar pattern of neuropathology that is particularly severe in the piriform and entorhinal cortices (Clifford et al., 1987; McDonough et al., 1998; Motte et al., 1998; Peredery et al., 2000). Finally, both Li-pilo (Jope et al., 1986; Morrisett et al., 1987) and soman convulsions (Shih et al., 1997; McDonough and Shih, 1997) are relatively refractory to drug therapy with the additional confound that the longer the SE continues the more difficult pharmacological treatment becomes (Walton and Treiman, 1988; Jones et al., 2002).

Propofol is a highly lipid soluble, nonbarbiturate anesthetic that possess anticonvulsant activity. Low doses disrupt ictal-like discharge in rat hippocampal slices (Rasmussen et al., 1996). In rodents, propofol inhibits seizures induced by electroshock, i.v. pentylenetetrazol (Lowson et al., 1990) and i.v. lidocaine (Lee et al., 1998). A single dose of 50 mg/kg propofol inhibits self-sustaining SE induced by perforant path stimulation in rats (Holtkamp et al., 2001). Propofol also has been found effective in humans as SE refractory to treatment with i.v. diazepam was controlled by propofol

(Kuisma and Roine, 1995). Propofol is comparable in efficacy to treatment with either high dose barbiturates (Stecker et al., 1998) or midazolam (Prasad et al., 2001) in human SE.

Numerous sedative hypnotic agents with agonist activity at the $GABA_A$ receptor ionophore complex have been used to arrest SE and lower the associated mortality rate in rats (Goodman, 1998). Although a $GABA_A$ agonist, propofol also acts to reduce NMDA-mediated whole cell currents (Orser et al., 1995) and is anticonvulsant in NMDA-induced seizures (Ahmad and Pleury, 1995). Propofol is also an antioxidant (Murphy et al., 1992; 1993; Tsuchiya et al., 2001) that may act as a neuroprotectant (Young et al., 1997; Gelb et al., 2002; Wang et al., 2002) by scavenging reactive oxygen species produced during SE. Because of these multiple properties propofol was deemed a viable candidate to test as a possible treatment for Li-pilo-induced SE and by extension as a treatment for organophosphorus nerve agent exposure.

2. Methods

2.1 *Animals*

Male, Sprague-Dawley rats obtained from Harlan (Indianapolis, IN) and weighing 290-325 g at the time of seizure test were used for these experiments. The animals were maintained in a climate-controlled vivarium at 21°C on a 12-hr light/dark cycle and allowed free access to food and water. All animal care and use conformed to the policies of the University of New Mexico Health Sciences Center.

2.2 *Intracranial implants*

The rats were anesthetized with equithesin (a mixture of chloral hydrate, pentobarbital, magnesium sulfate, ethanol, propylene glycol and water) for the surgical implantation of the electrocorticogram (ECoG) recording electrodes. Stainless steel screws were placed bilaterally in the skull 3 mm lateral to midline and equidistant between bregma and lambda. The screws were attached to connector pins by insulated wire. A third screw assembly was placed over the frontal sinus as a reference electrode. Additional screws were set in the skull to serve as anchors. All connector pins were inserted into a McIntyre connector (Ginder Scientific, Ottawa, ON). Screws, wires and connectors were secured in place with dental acrylic cement and the incision site closed

with surgical staples. Postoperative antibiotics (25,000 IU Durapen) and analgesics (0.02 mg/kg buprenorphine) were administered. Animals were allowed 7-10 days recovery before seizure testing.

2.3 *Seizure induction and ECoG recording*

The day prior to the seizure induction the rats were administered s.c. 3 mmol/kg lithium chloride (Sigma, St. Louis, MO) dissolved in normal saline. The lithium administration always preceded the pilocarpine administration by 20-24 h. The following day the animals were placed in a seizure observation cage and connected to a Grass Model 8 electroencephalograph by way of the implanted McIntyre connector for recording of ECoG. Pilocarpine (Sigma) dissolved in normal saline was administered s.c. in a dose of 25 mg/kg following 10 min of baseline ECoG recording. ECoG activity was recorded continuously throughout the experiment. To prevent lethal aspiration, atropine methylnitrate 10 mg/kg s.c. (Sigma) was administered 10 min prior to pilocarpine in those rats administered propofol immediately following pilocarpine (exposure treatment). SE was defined as the occurrence of continuous high amplitude ECoG spiking (Ormandy et al., 1989).

2.4 *Propofol administration*

Propofol was administered i.p. as the commercially available injectable emulsion Propoflo (Baxter Healthcare, New Providence, NJ). A 55 mg/kg dose of propofol was administered either immediately after pilocarpine administration (exposure treatment), following 5 min of SE or following 3 h of SE as defined by ECoG activity. An additional group was treated with 55 mg/kg propofol after 3 h SE followed 2 h later by 20 mg/kg propofol (extended treatment). Rats treated with lithium followed 20-24 h by s.c. normal saline injection as the vehicle control for pilocarpine followed 3 h later by propofol i.p. served as the seizure control group (No SE).

2.5 Histological preparation and digital imaging

Animals were sacrificed 24 h after pilocarpine administration as that is the period after which maximal SE-induced neuropathology is observed in Li-pilo convulsions (Clifford et al., 1987; Fujikawa et al., 1999). All animals were sacrificed by intraaortic perfusion-fixation while anesthetized with equithesin. The animals were initially perfused with heparinized phosphate buffered saline (PBS) (12.5 IU/ml, Sigma) followed

by 10% formalin PBS (VWR Scientific Products). Brains were removed and immersed in 10% formalin for a minimum of 24 h fixation. Following fixation the brains were paraffin embedded and sectioned into 5 μm sections by a rotary microtome (Microm International). Brain sections were mounted on glass slides and stained with hematoxylin and eosin (H&E).

Two consecutive sections were taken every 125 μm through the brain tissue 0.8 to 4.8 mm posterior to bregma (Paxinos and Watson, 1986). This specific brain region was chosen for analysis because it contains a preponderance of brain nuclei that exhibit the greatest degree of damage from soman (McDonough et al., 1998) and Li-pilo convulsions (Clifford et al., 1987; Motte et al., 1998; Fujikawa et al., 1999; Peredery et al., 2000). Of each pair of consecutive sections, one was used for independent histopathological analysis and the other section for optical density and lesion volume measurement.

Tissue sections used for histological analysis of were examined for the presence of necrosis and malacia using a scale of lesion severity developed for assessing soman toxicity (McDonough et al., 1989). The scale was as follows: 0 = none; 1 = minimal = $\leq$ 5% necrotic or malacic tissue; 2 = mild = 6-15% necrotic or malacic tissue; 3 = moderate =16-40% necrotic or malacic tissue; 4 = severe = >40% necrotic or malacic tissue. All sections were graded by a single observer (JG) in a blinded fashion. The median damage score from a given region across all tissue sections was used as the necrosis score for statistical tests (McDonough et al., 1998).

Tissue sections evaluated using optical density analysis were viewed using a stereomicroscope (Olympus BH2-RFCA). Images were acquired with a digital camera (Olympus MLH 020550) using the Olympus MagnaFire Camera Imaging and Control software (Version 1.1) and analyzed using Adobe Photoshop software (Version 5.0). An empirical parameter termed optical density was used as a measurement of necrotic and malacic tissue damage in piriform and perirhinal cortex. The average total light intensity in electronically defined (by hand) areas of equal size in both the piriform and perirhinal cortex was determined from digital images of the brain sections using the luminosity histogram function of the Adobe Photoshop software. This was compared by ratio to the average luminosity of identical electronically defined regions within the hypothalamus of the same section. Because the hypothalamus receives little or no damage in Li-pilo

convulsions (Clifford et al., 1987; Motte et al., 1998), the optical density in rats without SE was approximately 1 as the light intensity or luminosity in the cortical areas was similar to that in the hypothalamus. In rats with SE the optical density ratio was greater than 1.0 as the necrotic and malacic tissue in the piriform and perirhinal cortex allowed a greater transmission of light.

The tissue sections used for optical density analysis were also used for macroscopic lesion volume analysis. The same images acquired by stereomicroscope were analyzed by Image-Pro Plus software (Version 4.1). Digital images of identical magnification were taken of 11 comparable, consecutive sections from each rat and used for analysis. For each image, the area of the lesion was electronically defined by hand using the Image-Pro area measurement function and calculated as mm^2. The volume of the macroscopic lesion in each animal was determined as the product of the average area of the defined lesions in the consecutive sections and total distance between the sections.

2.6 Data and statistical analysis

Comparisons of propofol-induced termination of SE activity were made by student's t-test. Comparisons of the nonparametric histopathological rating score parameters were made using Mann-Whitney U test. Statistical comparison of optical density ratios between groups was determined by analysis of variance (ANOVA) followed by Newman-Kuels post hoc test when a significant difference ($P<0.05$) was determined by ANOVA. Values of $P<0.05$ were considered significant for all statistical tests.

3. Results

3.1 Propofol inhibition of Li-pilo SE

Pilocarpine administration in animals pretreated 20-24 h earlier with lithium induced SE with an average latency of 40 min (n=22, range 26 to 63 min). The average SE duration as defined by continuous high amplitude ECoG activity was 135 min (n=12, range 109-156 min).

Propofol had a significant impact on survival following 3 h of Li-pilo-induced convulsions. All rats treated with 55 (n=3) or 65 (n=3) mg/kg propofol i.p. after 3 h of SE survived the 24 h period following pilocarpine administration (Table 1). In contrast, only 3 of 6 animals survived the 24 h period when administered 50 mg/kg propofol.

Propofol completely inhibited all ECoG spiking activity when administered 3 h after onset of SE (Fig. 1). Propofol induced a complete suppression of ECoG spiking at which time the animals appeared anesthetized. Twenty-four h following pilocarpine administration the rats were ambulatory and exhibited interictal ECoG spiking (Fig. 1). Following the preliminary range finding study, all subsequent experiments that tested single doses of propofol used 55 mg/kg.

Propofol administered following 5 min of Li-pilo SE inhibited all ongoing seizure activity. The complete suppression of ECoG spiking required an average of 12.2 min (n=5) which was significantly less than the 20.8 min (t-test, $P<0.05$) required following 3 h SE which also completely suppressed all ECoG spiking (n=5). The rats appeared anesthetized when ECoG spiking was arrested. Propofol administered immediately following pilocarpine (exposure treatment) also induced an anesthetized state and prevented the onset of all seizure activity (n=5).

3.2 H&E Pathological Analysis

The histologic lesions, which were characterized at 24 hours after SE, were similar in distribution to previous reports and differed in the degree of severity of necrosis and malacia (Fig. 2). Necrosis was identified initially within neurons that had darkened nuclei and increased cytoplasmic eosinophilia. More severe lesions contained necrosis within adjacent glial cells, intercellular vacuolation and edema that was manifest as malacia with loss of tissue structure in the most severe lesions. Use of the grading scheme permitted an evaluation of the effectiveness of therapy that was consistent with previous reports. Necrosis scores of the piriform and perirhinal cortex of rats administered propofol either immediately following pilocarpine (exposure) or after 5 min of SE (5 min SE) did not differ significantly from that in animals not administered pilocarpine (No SE) (Fig. 3). The piriform and perirhinal regions in rats with only 5 min SE appeared indistinguishable from those not experiencing SE when viewed under low magnification (Fig. 4).

Li-pilo-induced convulsions of 3 h duration prior to propofol administration produced a significant degree of necrosis and malacia in the piriform and perirhinal cortex. As shown in Fig. 2, malacia was clearly visible in these critical regions. Necrosis scores of

these regions (Fig. 3) were significantly greater than in the No SE, exposure or 5 min SE groups (Mann-Whitney U test, $P<0.05$).

The propofol extended treatment group received 55 mg/kg propofol i.p. after 3 h SE followed 2 h later by an additional 20 mg/kg propofol i.p. The additional 20 mg/kg dose of propofol induced a striking decrease of necrosis and malacia obvious at both low magnification (Fig. 4) and from the necrosis scores derived from histologic examination at higher magnification (Fig. 3). The necrosis scores in the perirhinal cortex was significantly greater than that in the No SE, exposure and 5 min SE groups but was significantly less (Mann-Whitney U test, $P<0.05$) than that in the 3 h SE group which received only 55 mg/kg propofol (Fig. 3). The results were similar in the piriform cortex except that the difference between the 3 h SE group and the propofol extended treatment group did not reach statistical significance ($P=0.075$) in the Mann-Whitney U test.

3.3 Optical Density Analysis

Optical density analysis further demonstrated the neuroprotective effects of propofol. The optical density ratios in the piriform and perirhinal regions of rats in the No SE, exposure and 5 min SE groups were significantly less (ANOVA and Newman-Kuels post hoc, $P<0.05$) than those in rats with 3 h SE (Fig. 5). The optical density ratios of the 3 h SE group approached 1.5 (Fig. 5) indicating a significant 50% increase in light transmission through these severely damaged regions. In contrast, the optical density ratios in the propofol extended treatment group were significantly less than those of the 3 h SE group that received only a single administration of 55 mg/kg propofol. The optical density of the propofol extended treatment group did not differ significantly from the No SE, exposure or 5 min SE groups (Fig. 5).

3.4 Lesion Volume Analysis

Extended propofol treatment offered significant protection from malacia in the piriform and perirhinal cortex as determined by macroscopic lesion volume. The No SE, exposure and 5 min SE groups had no detectable macroscopic lesions (Figs. 4 and 6). The 3 h SE group experienced an average lesion volume of 6.8 mm^3 which was significantly greater (ANOVA and Newman-Kuels post hoc, $P<0.05$) than the 0.7 mm^3 of the propofol extended treatment group (Fig. 6). Three rats in the propofol extended treatment group had no evidence of a macroscopic lesion in either the piriform or

perirhinal cortex. The macroscopic lesion volume of the extended treatment group did not differ significantly from the No SE, exposure or 5 min SE groups.

4. Discussion

The most significant finding of this study was the neuroprotection induced by propofol. Rats treated with a single 55 mg/kg dose of propofol following 3 h SE exhibited a significant degree of neuropathology in the piriform and perirhinal cortices as reported previously (Honchar et al., 1983; Clifford et al., 1987; Motte et al., 1998; Peredery et al., 2000; Dube et al., 2000; Dube et al., 2001). Similarly treated rats administered an additional 20 mg/kg propofol 2 h following the initial 55 mg/kg dose exhibited a significant degree of neuroprotection within the perirhinal and piriform cortices. This effect may be due in part to anticonvulsant activity as reported for other $GABA_A$ receptor agonists that reduce ongoing SE and enhance survival (Lemos and Cavalheiro, 1995; Motte et al., 1998; Covolan and Mello, 2000; Fujikawa et al., 2000; Peredery et al., 2000; Andre et al., 2001; Glien et al., 2001). However, propofol has additional notable pharmacological properties as an NMDA antagonist (Orser et al., 1995; Ahmad and Pleuvry, 1995) and as an antioxidant (Murphy et al., 1992; 1993; Tsuchiya et al., 2001). This is a critical difference from other $GABA_A$ agonists given the predominate role of glutamate and NMDA receptors in both Li-pilo and organophosphorus nerve agent mediated SE (Ormandy et al., 1989; Walton and Treiman, 1991; McDonough and Shih, 1993; McDonough and Shih, 1997). It is hypothesized that the SE-induced NMDA receptor activation leads to an increased production of reactive oxygen species (Lafon-Cazal et al., 1993; Coyle and Puttfarcken, 1993; Michaelis, 1998) that mediate the SE neuropathology (Bruce and Baudry, 1995; Rong and Baudry, 1996; Rong et al., 1999; Peterson et al., 2002). We propose that propofol's NMDA antagonism would attenuate the NMDA receptor–induced excitotoxicity while the antioxidant activity suppresses neuronal destruction by the reactive oxygen species. In support of this hypothesis, propofol has been shown to be anticonvulsant against NMDA-induced convulsions in mice (Ahmad and Pleuvry, 1995) and to be neuroprotective in ischemic stroke by a proposed antioxidant mechanism (Young et al., 1997; Gelb et al., 2002; Wang et al., 2002).

Accumulating evidence supports a role for reactive oxygen species in the cholinergic SE-induced neuropathology of the piriform and perirhinal cortices. Pazdernik et al. (2001) reported a depletion of the endogenous antioxidant glutathione in the piriform cortex the first 24 h following soman-induced SE, suggesting an oxidative stress phase of the seizures. This is supported by an increased reactive oxygen species production in the piriform cortex during Li-pilo-induced SE as measured by dihydroethidium (Peterson et al., 2002). Additional evidence is provided by HU-211, an NMDA antagonist and antioxidant that induces significant neuroprotection in a model of ischemic stroke (Eshhar et al., 1995). HU-211 also induces significant neuroprotection in soman-induced seizures, reducing the macroscopic lesion volume in rat piriform and perirhinal cortices by 81-86% without affecting the ongoing seizure activity (Filbert et al., 1999). In the present study, propofol extended treatment reduced the macroscopic lesion volume approximately 89% in addition to terminating the ongoing SE. Propofol may represent a significant advancement in controlling both the SE and neuropathology of cholinergic convulsions.

The present study confirms and extends the previous understanding of Li-pilo convulsions. The failure to observe significant neuropathology following 5 min of Li-pilo-induced SE corroborates previous reports that 30-60 min of pilocarpine or Li-pilo SE is required to observe neuronal damage (Fujikawa, 1996; Lemos and Cavalheiro, 1995; Motte et al., 1998). In addition, propofol terminated ongoing SE in significantly less time (12.2 min) following 5 min of SE than following 3 h of ongoing convulsions (20.8 min). This supports previous assertions that longer lasting Li-pilo convulsions are more difficult to treat (Jobe et al., 1986; Morrisett et al., 1987; Walton and Treiman, 1988; Jones et al., 2002). $GABA_A$ agonists lose potency as Li-pilo SE progresses (Walton and Treiman, 1988; Jones et al., 2002) yet propofol inhibited all motor and electrographic activity after 3 h SE. Although this may have been a result of $GABA_A$ activity, it is possible that propofol's NMDA antagonist activity (Orser et al., 1995; Ahmed and Pleuvery, 1995) contributed to the anticonvulsant effect. In support of this hypothesis, the NMDA antagonist MK-801 is effective in reducing or terminating Li-pilo SE during the late, glutamatergic phase (Ormandy et al., 1989; Walton and Treiman, 1991).

Severe neuropathology was observed in the piriform and perirhinal cortices following 3 h SE as previously reported for Li-pilo convulsions (Honchar et al., 1983; Clifford et al., 1987; Motte et al., 1998; Peredery et al., 2000; Dube et al., 2000; Dube et al., 2001). Necrosis scores of H&E sections in the current study are comparable to those reported for soman-induced SE in rats (McDonough et al., 1998). Although the histopathology grading scheme scores the tissue for lesion severity, it does not take into account lesion volume. The macroscopic lesions observed in this study were the result of coalescing malacia the total volume of which was clearly affected by propofol. The optical density ratio and lesion volume analysis were used to quantify the extent of the macroscopic malacic lesions. By sampling the average luminosity in the damaged piriform and perirhinal regions and comparing by ratio to the undamaged hypothalamus, the enhanced light transmission through malacic tissue provided a measure of the most severe lesion distribution. While the lesion volume determination provided a more conventional analysis, both lesion assessment techniques produced similar results regarding the propofol inhibition of lesions resulting from cholinergic convulsions. Whether the observed neuroprotection translates to functional neuroprotection or protection from the development of spontaneous limbic seizures (Lemos and Cavalheiro, 1995; Dube et al., 2000; Dube et al., 2001; Glien et al., 2001) requires further study.

Propofol possess a spectrum of activity not observed in other treatments of Li-pilo-induced SE. Administered at the time of pilocarpine exposure propofol inhibits seizure onset. Electrographic and behavioral SE of 3 h duration is completely inhibited by a single 55 mg/kg i.p. dose. When propofol treatment is extended by an additional 20 mg/kg i.p. dose 2 h after the initial treatment, a significant neuroprotectant effect is induced. These properties would appear to represent a significant improvement over treatments currently employed to control cholinergic convulsions.

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Table 1. Propofol induced a dose-dependent increase in 24 h survival following Li-pilo SE. Propofol was administered i.p. 3 h after the onset of SE as defined by continuous high amplitude ECoG spiking.

Propofol (mg/kg)	N	Percent Survival
50	6	50%
55	3	100%
65	4	100%

Figure Legends

Figure 1. Representative ECoG tracings from a rat in Li-pilo-induced SE that was treated with 55 mg/kg propofol. Propofol was administered 3 h after the onset of SE as defined by continuous high amplitude ECoG spiking. An example of ECoG activity representative of SE is shown in the tracing labeled "30 min SE". Propofol suppressed all ECoG spiking as shown in the tracing labeled "30 min after propofol". Rats appeared anesthetized when the ECoG spiking was suppressed. Interictal spiking was present 24 h following pilocarpine administration as seen in the bottom tracing.

Figure 2. Representative examples of lesion grades and normal control brain. All photographs were taken at 120X original magnification of H&E stained brain. A: piriform cortex, minimal lesion (score = 1). Scale bar represents 100 μm. There are a few dark necrotic neuron nuclei with over 95% of the cells normal. B: piriform cortex, mild lesion (score = 2). Between 6-15% of the neuron nuclei are dark and shrunken and the cytoplasms are more distinct due to increased eosinophilia indicating necrosis. There is mild vacuolation. C: perirhinal cortex, moderate lesion (score = 3). Between 16-40% of the neurons are necrotic, which includes pyknosis, karyorrhexis, and loss of cytoplasmic detail. There is moderate vacuolation, some of which are coalescing. D: piriform cortex, severe lesion (score = 4). Greater than 40% of the neurons are necrotic. The nuclei are dark, shrunken and pyknotic. Affected cytoplasms have increased eosinophilia. There is marked coalescing vacuolation and malacia.

Figure 3. Comparison of neuronal damage in piriform and perirhinal cortex as determined by pathological ratings in H&E sections. Using necrosis scores the neuropathology in the 3 h SE and propofol extended treatment (Ext Txmt) groups was significantly greater than that of all other groups (1=significant difference from No SE, Exposure, 5 min SE groups, Mann Whitney U-test, $P<0.05$). In perirhinal cortex the neuropathology was significantly less in the propofol extended treatment group than the 3 hr SE group (2=significant difference from 3 hr SE, Mann-Whitney U-test, $P<0.05$).

Although similar, the difference between the 3 hr SE groups and propofol extended treatment groups in the piriform cortex did not reach statistical significance (Mann-Whitney U test, P=0.075).

Figure 4. Representative low magnification H&E sections of rat brains demonstrating the extent of Li-pilo-induced neuropathology and the neuroprotection induced by propofol. No lesion is visible in the brain of the rat that experienced 5 min of SE prior to propofol (5 minutes SE), the brain is indistinguishable from the control brain (No SE). An extensive neuronal lesion is clearly visible in the piriform and perirhinal cortex of the rat that experienced 3 h SE prior to 55 mg/kg propofol administration (3 hours SE). The rat in the extended treatment received 55 mg/kg propofol after 3 h SE followed 2 h later by 20 mg/kg propofol. With the additional 20 mg/kg dose being the only difference, the extended treatment rat exhibited a marked decrease in piriform and perirhinal neuropathology. PRh: perirhinal cortex, Pir: piriform cortex, BLA: basolateral amygdala. Scale bar equals 1 mm.

Figure 5. Comparison of neuronal damage in piriform and perirhinal cortex as determined by optical density ratio. Ratios of 1.0 indicate equal light transmission through temporal cortex tissue and the hypothalamus indicating little or no tissue damage. Ratios greater than 1.0 indicate a greater transmission of light through necrotic and malacic tissue in piriform and perirhinal cortex. The optical density ratio was significantly greater in the 3 h SE groups as compared to all others (1= significantly different from No SE, exposure (Exp), 5 min SE and extended treatment (Ext Txmt) groups, ANOVA and Newman-Kuels post hoc, $P<0.05$).

Figure 6. Comparison of neuronal damage in piriform and perirhinal cortex as determined by macroscopic lesion volume. When perirhinal macroscopic lesions occurred they were continuous with those in the piriform cortex. Therefore, lesions were considered in their entirety and not separated by region. The macroscopic lesion volume was significantly greater in the 3 h SE group as compared to all others (1=significantly

different from No SE, exposure (Exp), 5 min SE and extended treatment (Ext Txmt) groups, ANOVA and Newman-Kuels post hoc, $P<0.05$).

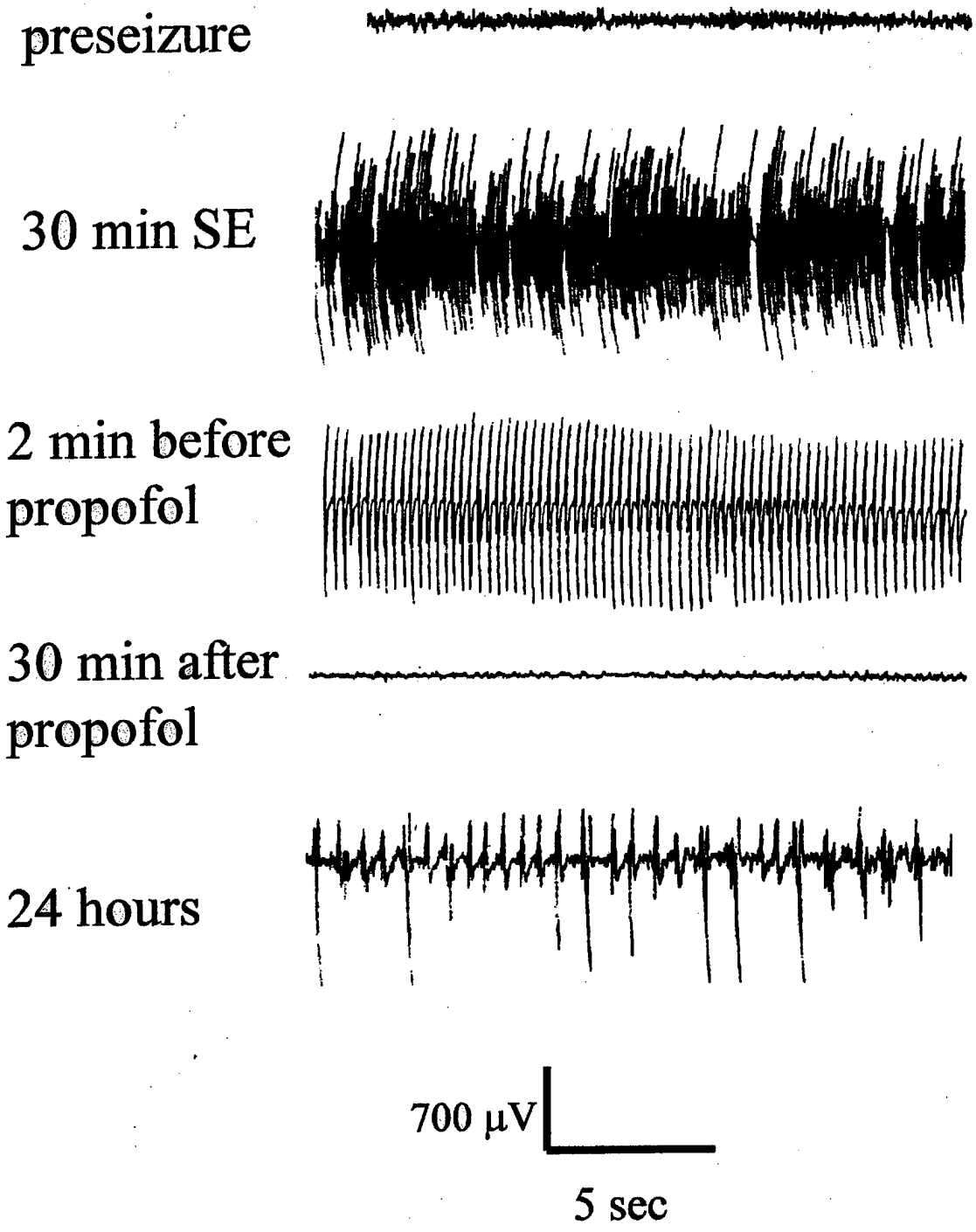
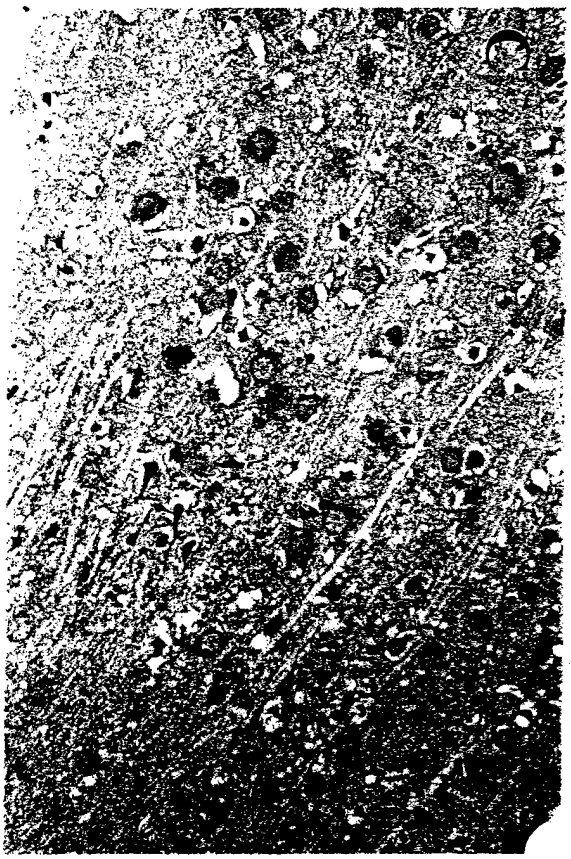
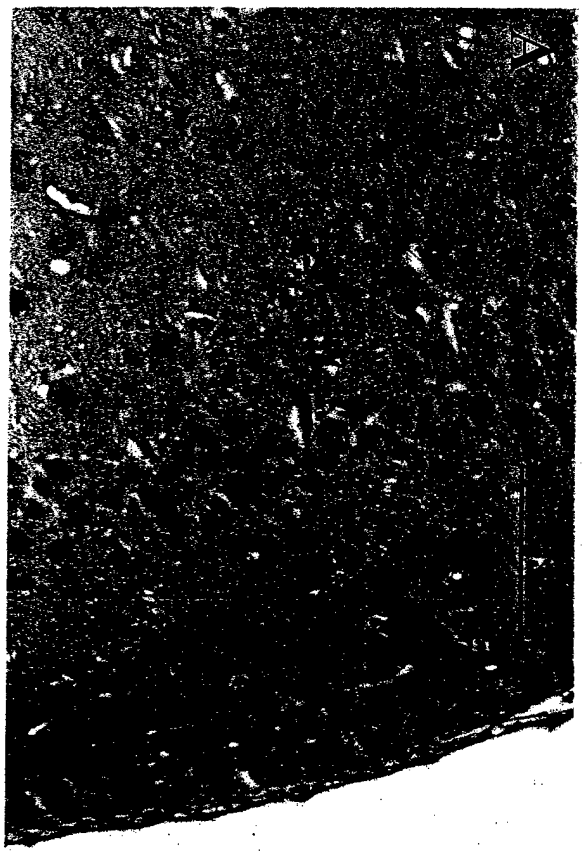

preseizure
30 min SE
2 min before propofol
30 min after propofol
24 hours
700 μV
5 sec

Figure 1





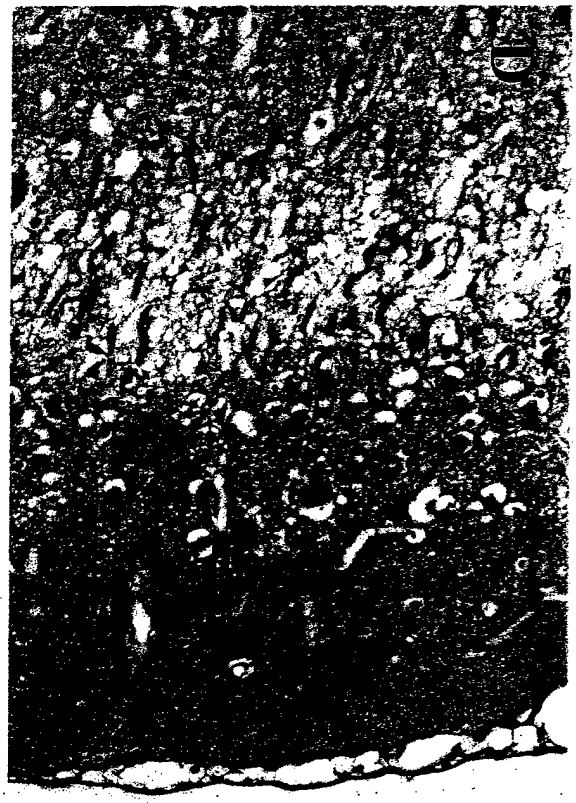


Figure 2

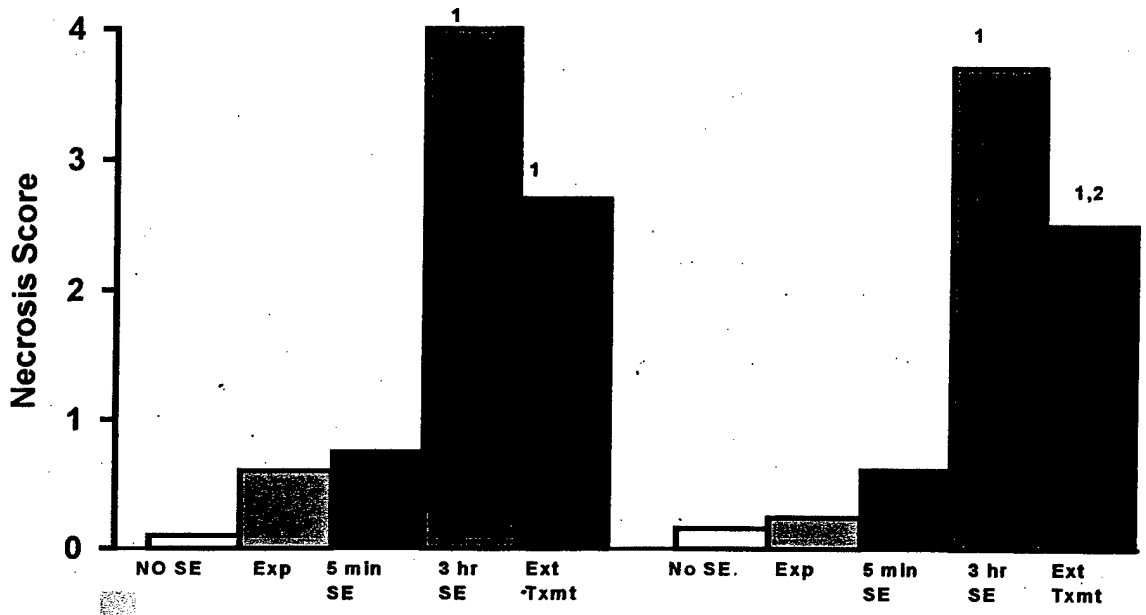
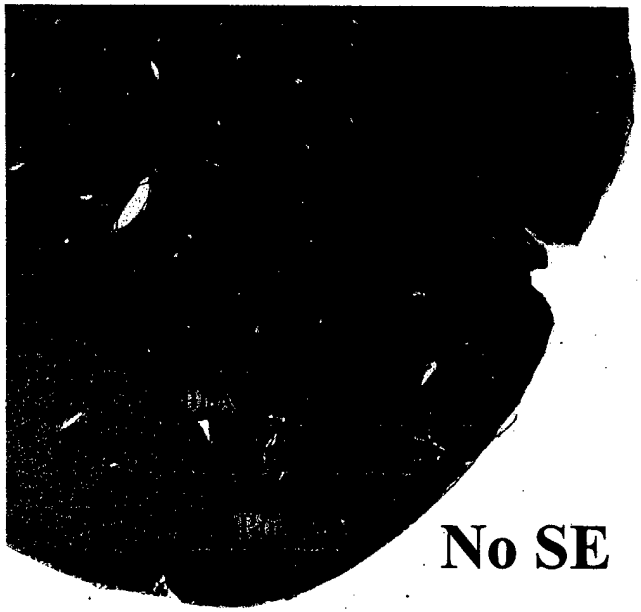
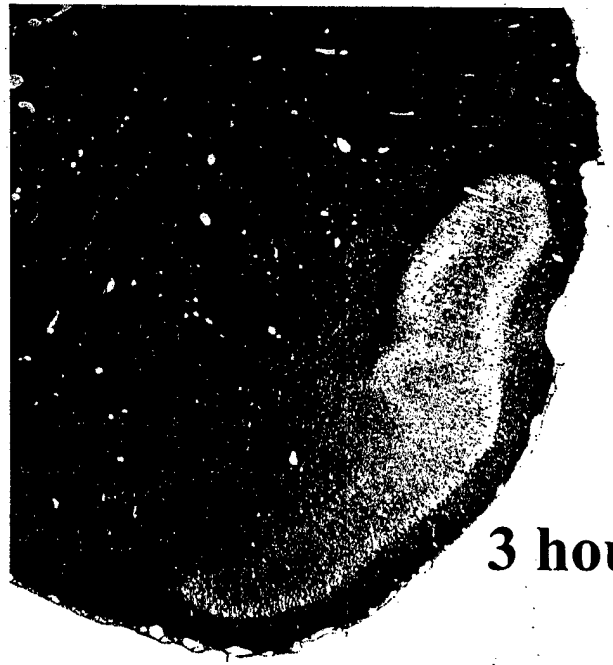

Necrosis Score
4
3
2
1
0
1
1
1
1,2
NO SE
Exp
5 min SE
3 hr SE
Ext Txmt
No SE.
Exp
5 min SE
3 hr SE
Ext Txmt
Piriform
Perirhinal

Figure 3

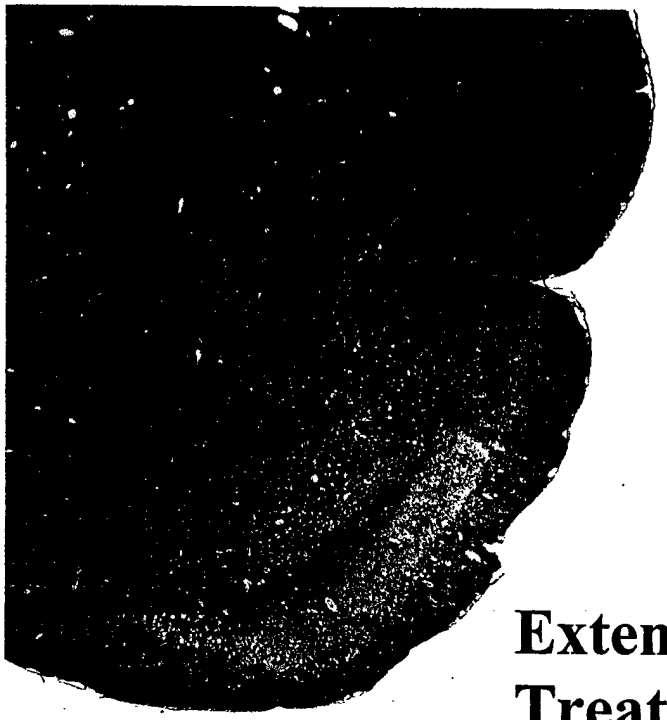


No SE



3 hours SE

5 minutes SE



Extended Treatment

Figure 4

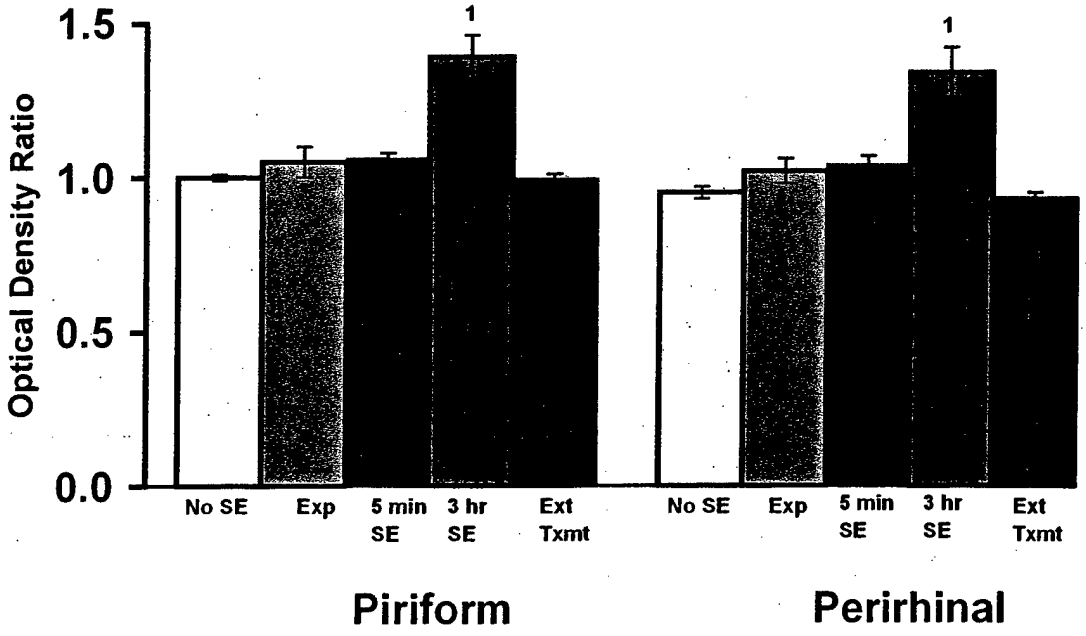

Optical Density Ratio
1.5
1.0
0.5
0.0
1
1
No SE
Exp
5 min SE
3 hr SE
Ext Txmt
No SE
Exp
5 min SE
3 hr SE
Ext Txmt
Piriform
Perirhinal

Figure 5

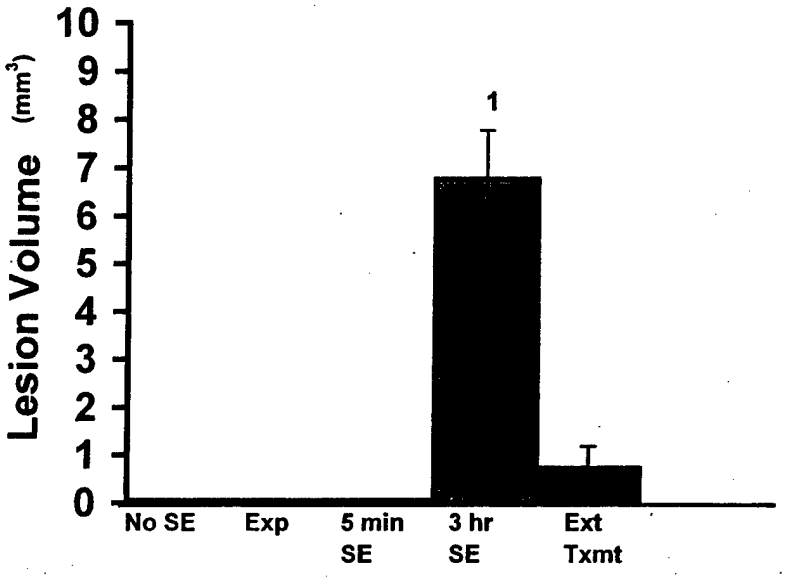

Lesion Volume (mm3)
10
9
8
7
6
5
4
3
2
1
0
1
No SE
Exp
5 min SE
3 hr SE
Ext Txmt

Figure 6

Differential Neuroprotective Effects of the NMDA Receptor-Associated Glycine Site Partial Agonists 1-Aminocyclopropanecarboxylic Acid (ACPC) and D-Cycloserine in Lithium-Pilocarpine Status Epilepticus

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Abstract

The status epilepticus (SE) induced in rats by lithium-pilocarpine (Li-pilo) shares many common features with soman-induced SE including a glutamatergic phase that is inhibited by NMDA antagonists. The present study determined whether 1-aminocyclopropanecarboxylic acid (ACPC) or D-cycloserine (DCS), both partial agonists of the strychnine-insensitive glycine site on the NMDA receptor ionophore complex, exerted anticonvulsant or neuroprotectant activity in Li-pilo SE. ACPC or DCS were administered either immediately following pilocarpine (exposure treatment) or 5 minutes after the onset of SE as determined by ECoG activity. SE was allowed to proceed for 3 hours before termination with propofol. The rats were sacrificed 24 hours following pilocarpine administration. Neither drug had an effect on the latency to seizure onset or the duration of seizure activity. ACPC administered 5 minutes after SE onset produced significant neuroprotection in cortical regions, amygdala and CA1 of the hippocampus. In contrast, when administered as exposure treatment ACPC enhanced the neural damage in the thalamus and CA3 of the hippocampus suggesting the neuropathology in those regions is mediated by a subset of NMDA receptors. DCS had no neuroprotectant activity in Li-pilo SE but exacerbated neuronal damage in the thalamus. Neither drug affected the cholinergic convulsions but had differential effects on neural damage. This suggests that the SE-induced seizure activity and subsequent neuronal damage involve independent mechanisms.

Keywords: D-Cycloserine; 1-Aminocyclopropanecarboxylic acid; Neuroprotection; Lithium; Pilocarpine; Status epilepticus

INTRODUCTION

Rats treated with lithium chloride 20-24 hours prior to pilocarpine administration develop status epilepticus (SE) of several hours duration (Honchar et al., 1983; Clifford et al., 1987). If allowed to proceed the SE produces significant neuropathology in the cortex, thalamus, hippocampus and amygdala regions (Honchar et al., 1983; Clifford et al., 1987; Motte et al., 1998; Peredery et al., 2000; Dube et al., 2000; Dube et al., 2001). Following a latent period of several weeks the animals that survive the SE develop spontaneous limbic seizures as a result of the neuropathology (Lemos and Cavalheiro, 1995; Dube et al., 2000; Dube et al., 2001; Glien et al., 2001).

Lithium-pilocarpine (Li-pilo) convulsions model the cholinergic convulsions induced by the organophosphorus nerve agent soman. Both Li-pilo (Jobe et al., 1986; Morrisett et al., 1987) and soman-induced SE (McDonough and Shih, 1993; Shih et al., 1997; McDonough and Shih, 1997) have an initial cholinergic phase that is inhibited by muscarinic antagonists. The SE induced by either Li-pilo (Ormandy et al., 1989; Walton and Treiman, 1991) or soman (Shih, 1990; McLean et al., 1992; McDonough and Shih, 1993; McDonough and Shih, 1997) have a secondary glutamatergic phase that is inhibited by glutamate antagonists (McDonough and Shih, 1997; Solberg and Belkin, 1997). Both chemoconvulsants produce a similar pattern of neuropathology that is particularly severe in the limbic system (Clifford et al., 1987; McDonough et al., 1998; Motte et al., 1998; Peredery et al., 2000).

NMDA receptor antagonists are effective anticonvulsants in both soman (Shih, 1990; McLean et al., 1992; McDonough and Shih, 1993) and Li-pilo convulsions (Ormandy et al., 1989; Walton and Treiman, 1991). Unfortunately both competitive and noncompetitive NMDA antagonists typically induce undesirable psychotomimetic adverse effects that have resulted in searches for alternative treatments (Rogawski, 1992; Lallement et al., 1997). One potential mechanism for alternative treatment might involve the strychnine-insensitive glycine receptor which is an allosteric glycine recognition site located on the NMDA receptor/ionophore complex (Bowery, 1987; Bonhaus et al., 1987). Glycine acts at this site as a required co-agonist (Kleckner and Dingledine, 1988) to facilitate the opening of the NMDA receptor cation channel in a strychnine-insensitive

manner (Johnson and Ascher, 1987). Antagonists of the strychnine-insensitive glycine site inhibit NMDA receptor activity (Kleckner and Dingledine, 1989; Kemp et al., 1988; Sircar et al., 1989) but also induce unacceptable levels of psychotomimetic activity (Rogawski, 1992). However, partial agonists of the strychnine-insensitive glycine site may inhibit NMDA receptor activity without inducing psychotomimetic activity (Rogawski, 1992; Walz, 1998; Karcz-Kubicha, 1999). As high efficacy agonists, partial agonists would have minimal psychotomimetic activity but would act as antagonists during periods of intense NMDA receptor activity such as that induced by cholinergic convulsions (McDonough and Shih, 1997; Solberg and Belkin, 1997; Nahum-Levy et al., 1999).

D-Cycloserine (DCS) is a partial agonist of the strychnine-insensitive glycine site with 40-70% the efficacy of glycine (McBain et al., 1989; Hood et al., 1989; Watson et al., 1990; Henderson et al., 1990; Emmett et al., 1991; Karcz-Kabicha et al., 1997). DCS induces anticonvulsant activity in maximal electroshock seizures (Peterson, 1992; Peterson and Schwade, 1993; Walz, 1998) and kindled amygdaloid seizures (Loscher et al., 1994; Rundfeldt et al., 1994) with minimal behavioral toxicity (Wlaz, 1998). DCS also induces significant reductions in the behavioral component of kainate seizures (Baran et al., 1994). Because of this anticonvulsant profile, DCS was deemed an ideal candidate to test the hypothesis of strychnine-insensitive glycine site partial agonists as anticonvulsants in Li-pilo SE.

1-Aminocyclopropanecarboxylic acid (ACPC) is also a high efficacy partial agonist of the strychnine-insensitive glycine receptor with 60-92% the efficacy of glycine (Marvizon et al., 1989; Watson and Lanthorn, 1990; Karcz-Kubicha et al., 1997). ACPC has anticonvulsant activity in NMDA-induced seizures in mice (Skolnick et al., 1989; Bisaga et al., 1993) and audiogenic seizures in genetically epilepsy-prone rats (Smith et al., 1993) with minimal behavioral toxicity (Smith et al., 1993; Skolnick et al., 1989). ACPC also possess neuroprotective activity in that it inhibits glutamate-induced cell damage in cerebellar granule cell cultures (Fossom et al., 1995; Boje et al., 1993), protects against ischemic cell damage in an experimental model of ischemic stoke (Fossom et al., 1995) and reduces neuronal damage in an experimental model of spinal cord injury (Long and Skolnick, 1994). Due to the anticonvulsant and neuroprotectant

properties, ACPC was also tested for activity in the Li-pilo model of cholinergic convulsions.

METHODS

Animals

These experiments used male, Sprague-Dawley rats obtained from Harlan (Indianapolis, IN) and weighing 290-325 g at the time of seizure test. The animals were maintained in a climate-controlled vivarium at 21°C on a 12-hr light/dark cycle with food and water available *ad libitum*. All animal care and use conformed to the policies of the University of New Mexico Health Sciences Center.

Intracranial implants

Rats were anesthetized with equithesin (a mixture of chloral hydrate, pentobarbital, magnesium sulfate, ethanol, propylene glycol and water) for the surgical placement of the electrocorticogram (ECoG) recording electrodes. Stainless steel ECoG recording screws were placed bilaterally in the skull 3 mm lateral to midline and equidistant between bregma and lambda. The screws were attached to connector pins by insulated wire. A third screw assembly was placed over the frontal sinus as a reference electrode and additional screws were set in the skull to serve as anchors. All connector pins were inserted into a McIntyre connector (Ginder Scientific, Ottawa, ON). Screws, wires and connectors were secured in place with dental acrylic cement and the incision site closed with surgical staples. Postoperative antibiotics (25,000 IU Durapen) and analgesics (0.02 mg/kg buprenorphine) were administered. Animals were allowed 7-10 days recovery before seizure testing.

Seizure induction and ECoG recording

The day prior to the seizure induction the rats were administered s.c. 3 mmol/kg lithium chloride (Sigma, St. Louis, MO) dissolved in normal saline. The lithium administration always preceded the pilocarpine administration by 20-24 hours. The following day the animals were placed in a seizure observation cage and connected to a Grass Model 8 electroencephalograph by way of the implanted McIntyre connector for recording of ECoG. Pilocarpine (Sigma) dissolved in normal saline was administered s.c. in a dose of 25 mg/kg following 10 minutes of baseline ECoG recording. ECoG activity

was recorded continuously throughout the experiment. SE was defined as the occurrence of continuous high amplitude ECoG spiking (Ormandy et al., 1989).

ACPC and DCS testing

ACPC (Sigma) and DCS (Sigma) were dissolved in saline and administered by i.p. injection in a volume of 4 ml/kg to assure adequate absorption (White et al., 1995). For the exposure treatment ACPC or DCS were administered immediately following the pilocarpine administration. For the 5 minute SE group the test drugs were administered 5 minutes after the onset of SE as determined by continuous high amplitude ECoG spiking.

Propofol administration

The ongoing SE was terminated by propofol administered i.p. as the commercially available injectable emulsion Propoflo (Baxter Healthcare, New Providence, NJ). A 55 mg/kg dose of propofol was administered following 3 hours of SE as defined by ECoG activity. Preliminary studies determined that this treatment terminated the Li-pilo SE and increased 24 hour survival to 100% without affecting the convulsion-induced neuropathology.

Spontaneous activity assessment

A computer controlled Rodent Activity Analyser (Omnitech Electronics) was used to determine spontaneous locomotor activity as a measure of seizure-induced neurological deficit (Fitzgerald, et al., 1988). The system included activity monitor cages (40.5X40.5X20 cm) with 2 sets of 16 photocells located at right angles to each other to record horizontal activity. The activity cages were located in light and sound attenuated chambers. The spontaneous locomotor activity was determined over two 10 min test periods. The preseizure test occurred 24 hours prior to pilocarpine and just before the lithium administration. The postseizure test occurred 24 hours after pilocarpine administration and just prior to brain perfusion-fixation. The parameters measured were distance traveled (DT) and resting time (RT). Activity on the postseizure test was expressed as a percent of the preseizure test activity.

Histological preparation and digital imaging

Animals were sacrificed 24 hours following pilocarpine administration as that is the period after which maximal SE-induced neuropathology is observed in Li-pilo convulsions (Clifford et al., 1987; Fujikawa et al., 1999). All animals were sacrificed by

intraaortic perfusion-fixation while anesthetized with equithesin. The animals were initially perfused with heparinized phosphate buffered saline (PBS) (12.5 IU/ml, Sigma) followed by 10% formalin PBS (VWR Scientific Products). Brains were removed 4 to 6 days following perfusion and immersed in 10% formalin for a minimum of 24 h of postfixation. Following postfixation the brains were paraffin embedded and sectioned into 5 μm sections by a rotary microtome (Microm International). Brain sections were mounted on glass slides and stained with hematoxylin and eosin (H&E).

Tissue sections were taken every 125 μm through the brain tissue 0.8 to 4.8 mm posterior to bregma (Paxinos and Watson, 1986). This specific brain region was chosen for analysis because it contains a preponderance of brain nuclei that exhibit the greatest degree of damage from soman (McDonough et al., 1998) and Li-pilo convulsions (Clifford et al., 1987; Motte et al., 1998; Fujikawa et al., 1999; Peredery et al., 2000). A scale of lesion severity developed for assessing soman toxicity (McDonough et al., 1989) was used to score the neuronal damage. The scale was as follows: 0 = none; 1 = minimal = $\leq$ 5% necrotic or malacic tissue; 2 = mild = 6-15% necrotic or malacic tissue; 3 = moderate =16-40% necrotic or malacic tissue; 4 = severe = >40% necrotic or malacic tissue. All sections were rated by a single observer (JG) in a blinded fashion. The mean damage score from a given region across all tissue sections was used as the neuronal damage score for statistical tests (McDonough et al., 1989).

Data and statistical analysis

Comparisons of the histopathological rating score parameters were performed using Kruskal-Wallis H-test for nonparametric statistical analysis. Statistical comparison of latency to SE onset, SE duration and spontaneous activity between groups was determined by analysis of variance (ANOVA) followed by Newman-Kuels post hoc test when a significant difference was determined by ANOVA. Values of $P<0.05$ were considered significant for all statistical tests.

RESULTS

ACPC and DCS effect on seizure activity

Neither ACPC nor DCS induced any significant anticonvulsant effect on Li-pilo-induced SE. The average latency to SE onset in vehicle control animals was 33.8

minutes. The duration of SE as determined by continuous high frequency ECoG spiking in vehicle control animals was 138.4 minutes. Neither drug had any significant effect (one way ANOVA) on the latency to SE when administered as exposure treatment immediately following the pilocarpine injection (Table 1). Likewise neither drug had any significant effect (one way ANOVA) on SE duration whether administered as exposure treatment or following 5 minutes of SE (Table 1).

ACPC and DCS effect on neurological deficit

Neither ACPC nor DCS had any significant effect on Li-pilo SE-induced neurological deficit as determined by spontaneous activity. The average distance traveled in the postseizure trial was in the range of 22-31% of the preseizure distance in vehicle control rats. The average resting time in the postseizure trial ranged from 199-220% of the preseizure time in vehicle control rats. The decreased activity 24 hours following pilocarpine administration was not significantly affected (one way ANOVA) by either ACPC or DCS whether administered as exposure treatment or 5 minutes after SE onset (Table 2).

ACPC and DCS effect on neuropathology

As indicated in Table 3, the brain structures in the vehicle control groups demonstrated mean neural damage scores of 1.34 to 4.0 corresponding from 6% to greater than 40% necrotic or malacic tissue. These data indicate that the Li-pilo model of soman-induced SE in our hands induces a quantifiable degree of neuronal damage in the expected brain structures (Clifford et al., 1987; McDonough et al., 1989; McDonough et al., 1998; Fujikawa, 1999). These data also demonstrate that the 55 mg/kg dose of propofol administered to limit SE and improve 24 hour survival, had no effect on the neuropathology induced by 3 hours of Li-pilo seizures.

Significant neuroprotection was observed when ACPC was administered 5 minutes after the onset of Li-pilo-induced SE. The neuroprotection was most obvious in the temporal lobe regions as the macroscopic lesions induced by Li-pilo-induced SE were clearly reduced by ACPC administration (Fig. 1). As indicated in Table 3 and Figure 2, both ACPC doses administered 5 minutes after the onset of SE significantly (Kruskal-Wallis H-test) reduced the neuropathology in all areas of the cortex as well as the cortical and lateral amygdala nuclei and amygdalopiriform regions. The differences between the

2 ACPC dose groups did not differ significantly. The 200 mg/kg ACPC dose also reduced the neuronal damage in the medial amygdala. Histologically brain regions from the vehicle control animals demonstrated dark basophilic shrunken nuclei with increased darkened cytoplasmic eosinoplilia and vacuolation (Fig. 3). In areas affected by ACPC treatment 5 minutes after SE onset, the few affected cells contained darkened, occasionally angulated nuclei and slightly increased cytoplasmic eosinophilia and vacuolation (Fig. 3). Activity in the hippocampus was mixed with neuroprotection by 200 mg/kg ACPC in the CA1 but a significant increase by neuropathology ratings in the CA3 by the 100 mg/kg dose (Table 3, Fig. 2).

ACPC exposure treatment induced little neuroprotective activity but appeared to exacerbate the Li-pilo-induced neuropathology in regions of the thalamus and hippocampus. ACPC exposure treatment (administered immediately following pilocarpine) induced neuroprotection only in the perirhinal cortex at the 200 mg/kg dose (Table 3). In contrast, both doses of ACPC administered as exposure treatment significantly enhanced neuronal damage (Kruskal-Wallis H-test) in the mediodorsal thalamus, laterodorsal thalamus, pretectal nucleus and the hippocampal CA3 region (Table 3, Fig. 4). The enhanced neurodamage was observed as an increase in the number of necrotic neurons with dark basophilic shrunken and sometimes angulated nuclei with increased cytoplasmic eosinophilia and vacuolation (Fig. 5).

Treatment with DCS either at exposure or following 5 minutes SE had no significant effect on the Li-pilo-induced neuropathology in most brain regions (Table 3). The exceptions were the mediodorsal thalamus and pretectal nucleus in the 125 mg/kg DCS exposure treatment group in which the neuropathology was significantly greater (Kruskal-Wallis H-test) than the vehicle control group (Table 3, Figure 4). The appearance of the lesions in the mediodorsal thalamus and pretectal nucleus associated with DCS were histologically similar to those induced by ACPC as illustrated in Figure 5.

DISCUSSION

ACPC produced significant neuroprotection in Li-pilo convulsions when administered 5 minutes following SE onset. ACPC reduced by half the mean neural damage scores

induced by 3 hour SE in the parietal, occipital and perirhinal cortices. Significant reductions were also produced in the piriform cortex, amygdala and CA1 region of the hippocampus. This collaborates previous reports of ACPC neuroprotection in other experimental models including primary neuronal cell cultures (Boje et al., 1993; Fossom et al., 1995), cerebral ischemia (Fossom et al., 1995) and dynorphin A-induced spinal injury (Long and Skolnick, 1994). The neuroprotection resulted from a single ACPC administration whereas the neuroprotection in Li-pilo SE by vigabatrin (Andre et al., 2001) or caffeine (Rigoulot et al., 2003) required chronic administration. Given that the duration of pharmacological activity following i.p. administration in rats is approximately 1 hour (Smith et al., 1993), we predict that repeated ACPC administrations would produce even greater neuroprotection during the 3 hour duration SE that was tested in this experiment. The brief duration of action would also explain the lack of neuroprotection when ACPC was administered as exposure treatment. Finally, ACPC was neuroprotective without affecting ongoing seizure activity. Pharmacologically induced neuroprotection without anticonvulsant activity has been reported in Li-pilo SE (Andre et al., 2001; Rigoulot et al., 2003), kainic acid SE (Rong et al., 1999) and soman SE (Filbert et al., 1999). This suggests that the mechanisms of SE-induced seizure activity and neuronal damage are linked but independent.

In contrast to ACPC, DCS had no neuroprotective activity in Li-pilo-induced SE. Both DCS (Hood et al., 1989; Henderson et al., 1990; Watson et al., 1990) and ACPC (Marvizon et al., 1989; Watson and Lanthorn, 1990) were originally identified as high efficacy partial agonists of the strychnine-insensitive glycine site. However, clear differences between the 2 drugs in anticonvulsant activity (Bisaga et al., 1993; Peterson, 1995), anxiolytic activity (Karcz-Kubicha et al., 1997) and neuroprotection in cell culture (Boje et al., 1993; Widdowson et al., 1996) have been reported in addition to the differential *in vivo* neuroprotection observed in this study. Additional differences in the mechanisms of pharmacological activity have been identified using NMDA receptors expressed in *Xenopus* oocytes where ACPC has been shown to be both a full agonist of the strychnine-insensitive glycine site and a low affinity competative NMDA antagonist (Nahum-Levy et al., 1999). The degree of interaction between the opposing ACPC effects depends on the subunit composition of the NMDA receptors in a particular region

(Sheinin et al., 2002). In contrast, DCS acts only at the glycine site but may have either greater or less efficacy than glycine depending on the NMDA receptor subunit composition (Sheinin et al., 2001). These findings indicate that glutamate antagonism is critical for the ACPC-induced neuroprotection while specific activity at the glycine site, as is the case for DCS, provides no neuroprotection in Li-pilo SE.

ACPC produced significant neuroprotection in the cortical and amygdala regions. The competitive NMDA antagonist CGP 40116 (Fujikawa et al., 1994) and noncompetitive NMDA antagonist ketamine (Fujikawa, 1995) also produced significant neuroprotection in cortical areas in Li-pilo SE. In contrast, caffeine (Rigoulet et al., 2003) and vigabatrin (Andre et al., 2001) either had no neuroprotective effect or exacerbated Li-pilo neuronal damage in cortical areas. Taken together the evidence would suggest that NMDA antagonism is necessary for cortical neuroprotection. The predominance of NMDAR2A receptors in cortical areas (Ishii et al., 1993) where ACPC has greater competitive NMDA antagonist activity (Sheinin et al., 2002) may explain the ACPC neuroprotection observed there.

Although neuroprotective in cortical and amygdala regions when administered 5 minutes after SE onset, ACPC administered as exposure treatment exacerbated the neuropathology observed in thalamic regions. DCS also increased neuronal damage in the thalamus when administered as exposure treatment. We hypothesize that the variable effect of ACPC and DCS on neuropathology is due to regional differences in NMDA receptor subtype distribution (Ishii et al., 1993; Laurie et al., 1995; Goebel and Poosch, 1999). For example, the enhanced thalamic neuropathology may result from the presence of NMDAR2B and 2C receptors (Ishii et al., 1993) where ACPC exerts less competitive NMDA antagonist and more glycine agonist activity that would enhance NMDA receptor excitotoxicity (Nahum-Levy et al., 1999; Sheinin et al., 2002). The thalamus also contains a significant proportion of NMDAR2C receptors (Ishii et al., 1993) where DCS exerts greater than full agonist activity at the strychnine insensitive glycine sites thereby facilitating excitotoxicity in that region (Newell et al., 1997; Sheinin et al., 2001). Interestingly, the enhanced neuropathology following ACPC exposure treatment, but not following SE onset administration, suggests different populations of NMDA receptors are involved at different times during SE.

ACPC administered after 5 minutes of SE or as exposure treatment potentiated the neuropathology in CA3 (Fig. 2 and 4) but was neuroprotective in CA1 (Fig. 2). Nonuniform CA3 and CA1 regional responses to neuroprotective drug treatments have also been reported following caffeine (Rigoulot et al., 2003) and vigabatrin (Andre et al., 2001) treatment in Li-pilo seizures. This evidence supports a hypothesis of regional differences in the mechanism of neuropathology in Li-pilo-induced SE (Peterson et al., 2002; Rigoulot et al., 2003). In the case of ACPC the regional responses may be related to the distribution of hippocampal NMDA2A and 2B receptors (Ishii et al., 1993) where ACPC has variable degrees of activity as both a glycine site agonist and competative NMDA antagonist (Newell et al., 1997; Sheinin et al., 2002).

The reduced spontaneous motor activity observed 24 hours following pilocarpine administration was not affected in rats demonstrating significant ACPC neuroprotection. This supports a previous report that morphological neuroprotection is not well correlated with recovery of behavioral activities when tested immediately following pilocarpine-induced SE (Hort et al., 1999). The onset of spontaneous seizures also is used as a measure of behavioral or functional neuroprotection. Significant levels of morphological neuroprotection induced by pharmacological agents or kindled amygdala seizures did not affect the onset of spontaneous seizures following Li-pilo-induced SE (Andre et al., 2000; Andre et al., 2001; Rigoulot et al., 2003) or in kainic acid-induced SE (Ebert el al., 2002). Further studies are required to determine effects of ACPC-induced neuroprotection on long-term cognitive function and the development of spontaneous seizures following Li-pilo SE.

DCS inhibits kainic acid-induced convulsions (Baran et al., 1994). In the present study DCS doses in the same range as those effective in kainic acid convulsions (Baran et al., 1994) had no anticonvulsant or neuroprotective effect in Li-pilo-induced SE. This discrepancy may be a result of the present study evaluating multiple parameters of SE activity including electrographic ECoG seizure activity, latency to SE, SE duration and neuropathology while the kainate study evaluated only behavioral convulsions (Baran et al., 1994). Alternatively, the differential DCS effect may result from fundamental differences in the mechanism of kainic acid and Li-pilo-induced seizures. Kainic acid is hypothesized to act presynaptically to induce glutamate release (Ferkany et al., 1982;

Lehmann et al., 1983; Chittajally et al., 1996; Liu et al., 1997) while pilocarpine and other cholinergic convulsants activate muscarinic receptors that induce glutamate release (Jobe et al., 1986; Ormandy et al., 1989; McDonough and Shih, 1997; Solberg and Belkin, 1997). Because glutamate antagonists inhibit both kainic acid (Clifford et al., 1990; Berg et al., 1993) and Li-pilo convulsions (Ormandy et al., 1989; Walton and Treiman, 1991) we propose that different subsets of NMDA receptors are involved in the 2 seizure types and that DCS is active only with NMDA receptors involved in kainic acid SE (Ishii et al., 1993; Sheinin et al., 2001).

In conclusion, ACPC induced significant neuroprotection when administered at the onset of Li-pilo-induced SE. Due to a short duration of action, we propose that repeated ACPC doses would enhance the neuroprotective activity. The observed effects were nonuniform, with neuroprotection in the amygdala and cortical regions and an exacerbation of neuronal damage in the thalamus. The differential effect on neural damage is proposed to result from regional differences in NMDA receptor subunit composition that are variably affected by ACPC. The heterogeneity of NMDA receptor expression may also explain the lack of activity by DCS. Further studies are required to determine the effects of ACPC neuroprotection on the sequela of Li-pilo SE and how those effects may relate to the treatment of organophosphorous nerve agent exposure.

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Table 1 **Effect of ACPC and DCS on seizure activity**

Exposure Treatment

	control(n=8)	100 ACPC(n=7)	200 ACPC(n=8)	125 DCS(n=9)	250 DCS(n=7)
Latency to SE(mins)	33.8+/-1.5	37.6+/-2.9	32.5+/-1.58	38.0+/-2.8	46.7+/-5.9
Duration of SE(mins)	138.6+/-4.6	137.1+/-11.0	148.3+/-4.4	128.2+/-4.4	127.0+/-8.0

5 minute SE

	control(n=9)	100 ACPC(n=7)	200 ACPC(n=8)	125 DCS(n=9)	250 DCS(n=9)
Latency to SE(mins)	n/a	n/a	n/a	n/a	n/a
Duration of SE(mins)	125.4+/-2.7	135.6+/-8.9	145.0+/-6.8	133.3+/-6.3	134.0+/-3.7

Table 2 **Effect of ACPC and DCS on spontaneous behavior**

Exposure Treatment

	control(n=8)	100 ACPC(n=7)	200 ACPC(n=8)	125 DCS(n=9)	250 DCS(n=7)
Distance traveled	22.4+/-7.1	54.7+/-20.2	68.1+/-17.7	37.0+/-15.8	26.6+/-11.6
Resting time	219.5+/-22.3	185.0+/-37.0	169.3+/-35.5	212.7+/-31.1	256.3+/-42.6

5 minute SE

	control(n=9)	100 ACPC(n=7)	200 ACPC(n=8)	125 DCS(n=9)	250 DCS(n=9)
Distance traveled	31.4+/-8.8	47.0+/-15.6	78.9+/-23.4	52.0+/-20.0	53.0+/-10.5
Resting time	199.3+/-28.3	199.9+/-47.6	146.6+/-33.6	192.0+/-40.1	167.3+/-37.5

Table 3 **Effects of ACPC and DCS on neural damage**

Brain Region	Exposure Treatment				
	Control(n=8)	100 ACPC(n=7)	200 ACPC(n=8)	125 DCS(n=9)	250 DCS(n=7)
parietal cortex (Par1)	2.65	2.43	1.93	2.92	2.95
occipital cortex (Oc)	2.69	2.21	1.74	2.54	2.59
perirhinal cortex (PRh)	3.69	2.79	2.53*	3.74	3.61
piriform cortex (Pir)	4.00	3.38	3.51	4.00	4.00
cortical amygdala (Co)	3.91	3.71	3.57	3.83	3.68
medial amygdala (BM)	3.16	3.14	3.01	3.26	3.31
lateral amygdala (La)	3.58	3.46	3.51	3.89	3.85
mediodorsal thalamus (MD)	2.25	3.49*[a]	2.96*[a]	2.93*[a]	2.60
lateraldorsal thalamus (LD)	2.25	3.33*[a]	2.97*[a]	2.87	2.58
pretectal nucleus (APTD)	2.13	2.90*[a]	2.62*[a]	2.99*[a]	2.39
CA3	2.59	3.35*[a]	3.02*[a]	2.66	2.41
CA1	1.34	1.22	1.25	1.27	1.03
amygdalopiriform (Apir)	4.00	3.98	3.82	4.00	4.00

	5 minute SE				
	control(n=9)	100 ACPC(n=7)	200 ACPC(n=8)	125 DCS(n=9)	250 DCS(n=9)
parietal cortex (Par1)	3.00	1.50*	1.29*	2.54	2.54
occipital cortex (Oc)	2.80	1.32*	1.30*	2.44	2.40
perirhinal cortex (PRh)	3.59	1.89*	1.86*	3.16	3.42
piriform cortex (Pir)	3.97	3.16*	2.85*	3.83	3.98
cortical amygdala (Co)	4.00	3.30*	3.44*	3.97	3.94
medial amygdala (BM)	3.44	3.03	2.54*	3.44	3.43
lateral amygdala (La)	3.76	3.36*	3.04*	3.73	3.81
mediodorsal thalamus (MD)	3.09	2.87	2.52	2.94	2.89
lateraldorsal thalamus (LD)	3.20	2.98	2.53	3.05	3.12
pretectal nucleus (APTD)	2.74	2.74	2.10	2.79	2.66
CA3	2.81	3.32*[a]	2.89	2.79	2.72
CA1	1.46	1.09	0.94*	1.24	1.28
amygdalopiriform (Apir)	4.00	3.26*	3.63*	3.97	4.00

* indicates statistically significant difference from control group as determined by Kruskal-Wallis H-Test
[a] indicates that neuronal damage was enhanced by ACPC or DCS treatment

Figure Legends

Figure 1. H&E stained sections of the rat temporal lobe showing the macroscopic lesions resulting from Li-pilo-induced convulsions and the neuroprotection induced by ACPC administered 5 minutes after the onset of SE. PRh: perirhinal cortex, Pir: piriform cortex, BLA: basolateral amygdala. Scale bar represents 1 mm.

Figure 2. Graphic presentation of the neuroprotection induced by ACPC when administered 5 minutes after the onset of SE. Considerable reduction of neuropathology was observed in the parietal, occipital and perirhinal cortices regardless of the dose. Although more resistant to the single dose ACPC effects, neuroprotection was also observed in the amygdala and piriform cortex regions. Note that neuronal damage was enhanced in CA3 by the 100 mg/kg ACPC dose. * indicates significant difference from the corresponding vehicle control group as determined by Kruskal-Wallis H-test. Abbreviations are as indicated in Table 1.

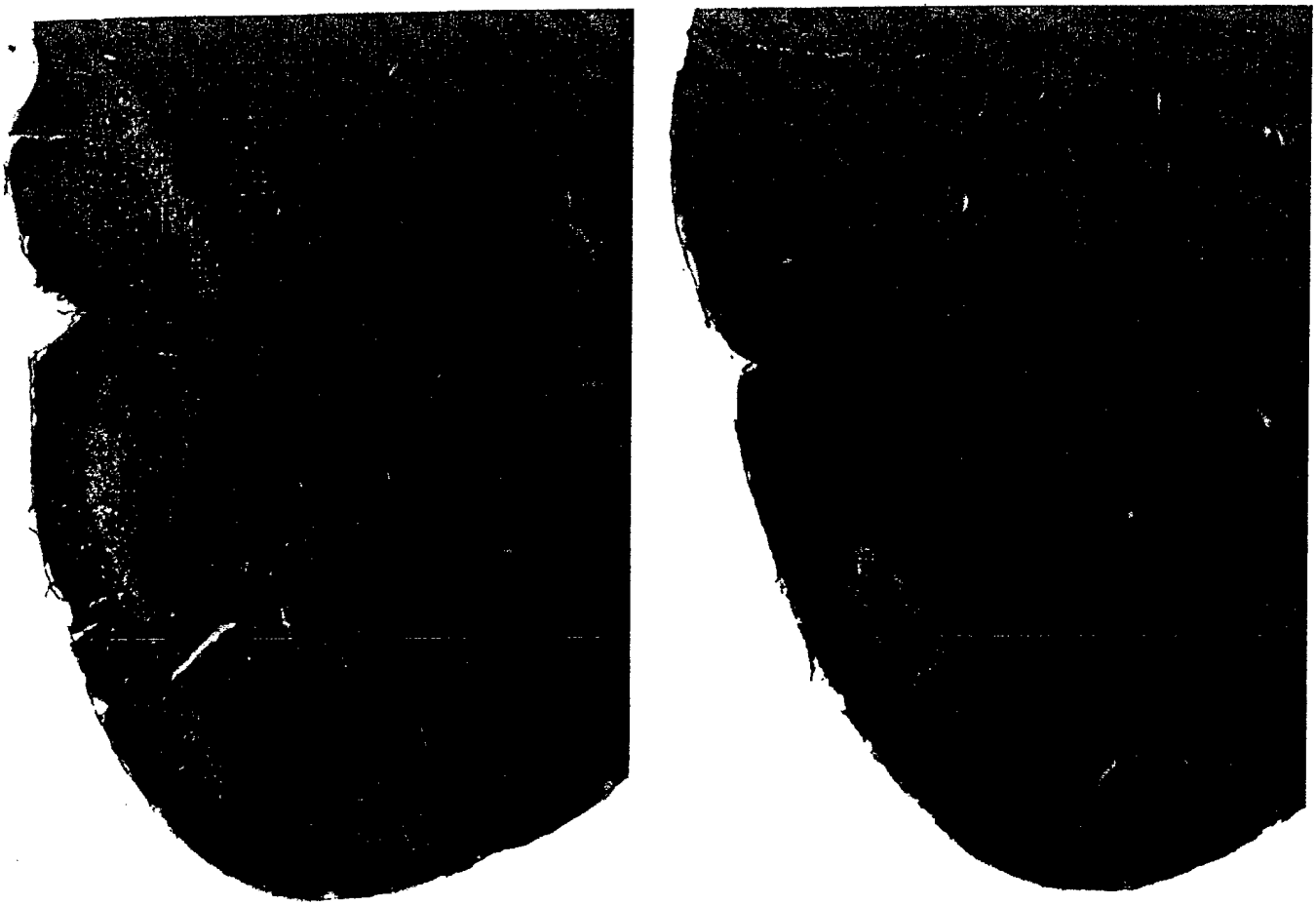
Figure 3. Representative high magnification H&E sections of rat brains showing the severity of Li-pilo-induced neuropathology and examples of the neuroprotection induced by ACPC. (a) Parietal cortex from control showing moderate necrosis of cortical neurons (16-40% cells necrotic). The affected neurons contain dark basophilic shrunken angulated nuclei with increased darkened cytoplasmic eosinophilia and vacuolation (200 X). (b) Parietal cortex from ACPC treatment begun 5 minutes after SE showing minimal necrosis of cortical neurons (1-5% cells necrotic). The few affected cells contain dark shrunken nuclei with increased darkened cytoplasmic eosinophilia and vacuolation (200X). (c) Perirhinal cortex from control showing severe necrosis of cortical region (> 40% cells necrotic). The affected cells are pyknotic and karyorrhectic with loss of cytoplasmic detail. Vacuolation, intercellular edema and malacia are extensive paralleling the adjacent sulcus (100 X). (d) Perirhinal cortex from ACPC treatment begun 5 minutes after SE showing minimal necrosis of neurons (1-5% cells necrotic). The few affected cells contain darkened occasionally angulated nuclei, slightly increased cytoplasmic eosinophilia and vacuolation (100 X). (e) Lateral amygdala from control showing severe necrosis (> 40% cells necrotic). The affected cells contain dark shrunken

nuclei and increased cytoplasmic eosinophilia or loss of cytoplasmic detail. Vacuolation, intercellular edema, malacia and occasional segmented leukocytes were also visible (200X). (f) Lateral amygdala from ACPC treatment begun 5 minutes after SE showing moderate necrosis (16-40% cells necrotic). The necrotic cells contain shrunken basophilic nuclei and the cytoplasms contain increased eosinophilia and vacuolation. The clear areas between cells indicate intercellular edema (200X). (g) Amygdalopiriform from control showing severe necrosis (> 40% cells necrotic). The affected cells contain dark pyknotic nuclei with increased eosinophilia and vacuolated cytoplasms. There is also intercellular edema and early malacia evident (100X). (h) Amygdalopiriform from ACPC treatment began 5 minutes after SE showing moderate necrosis (16-40% cells necrotic). The necrotic cells contain dark shrunken nuclei and increased eosinophilia and vacuolation of cytoplasm (100X).

Figure 4. Graphic presentation of the exacerbated neuronal damage induced by ACPC or DCS when administered as exposure treatment. Both ACPC and DCS enhanced neuropathology observed in the thalamus. The enhanced neuronal damage in lateraldorsal thalamus (LD) by DCS did not reach statistical significance. ACPC also enhanced neuronal damage in CA3. * indicates significant difference from the corresponding vehicle control group as determined by Kruskal-Wallis H-test. Abbreviations are as indicated in Table 1.

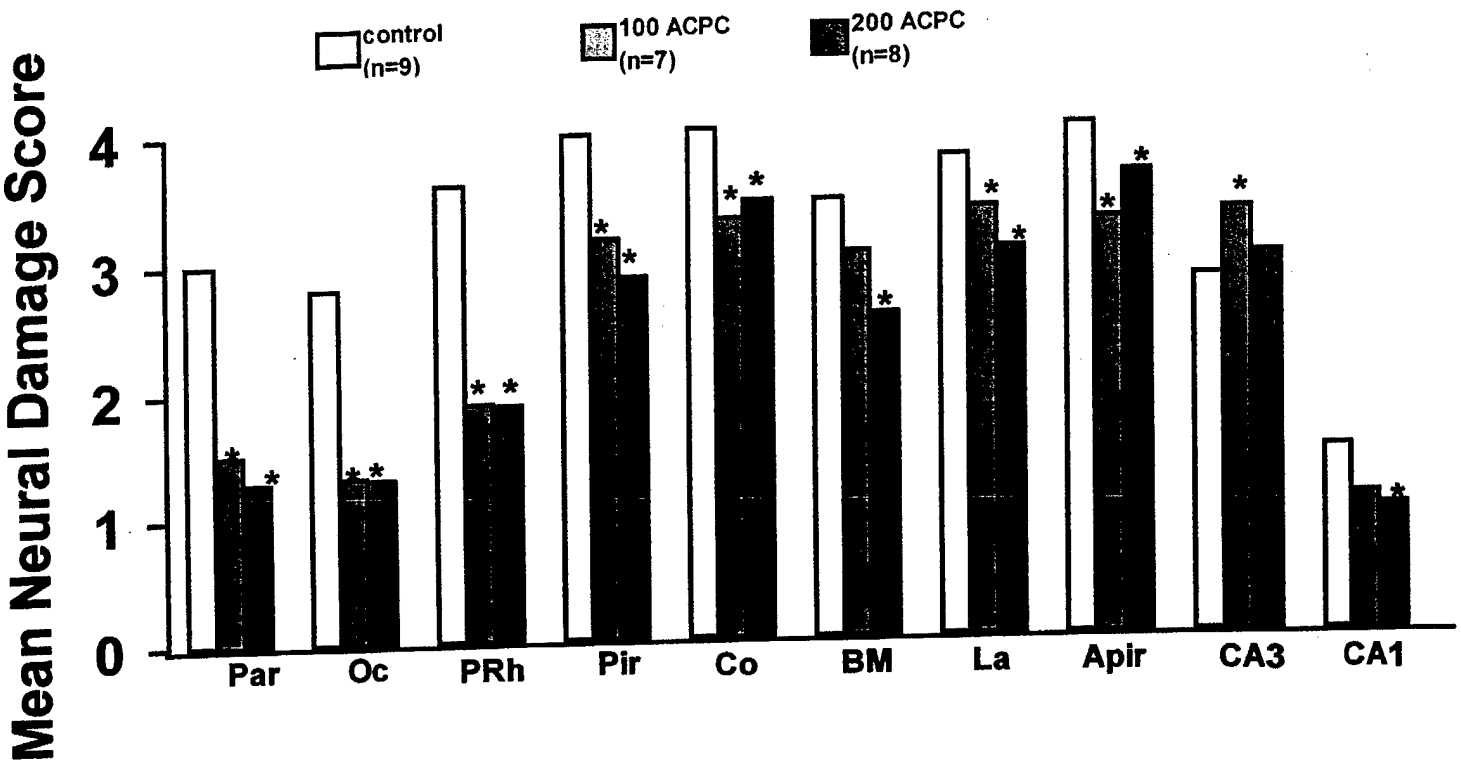
Figure 5. Representative H&E sections of rat brains demonstrating the enhancement of Li-pilo-induced neuropathology by ACPC and DCS. (a) Mediodorsal thalamus from control showing mild necrosis (6-15% cells necrotic). The affected neurons contain basophilic slightly shrunken angulated nuclei with increased cytoplasmic eosinophilia and vacuolation (200X). (b) Mediodorsal thalamus from ACPC exposure treatment showing severe necrosis (>40% cells necrotic). The affected neurons contain dark basophilic shrunken sometimes angulated nuclei with increased cytoplasmic eosinophilia, vacuolation and loss of cytoplasmic detail (200X). (c) Pretectal nucleus from control showing mild necrosis (6-15% cells necrotic). The necrotic cells contain shrunken basophilic nuclei with increased cytoplasmic eosinophilia and vacuolation (200X). (d)

Pretectal nucleus from ACPC treatment showing severe necrosis (>40% cells necrotic). The necrotic cells contain shrunken basophilic angulated nuclei with increased cytoplasmic eosinophilia and vacuolation (200X). (e) CA3 from control showing moderate necrosis (16-40% cells necrotic). The necrotic cells contain shrunken basophilic nuclei and increased cytoplasmic eosinophilia and vacuolation (200X). (f) CA3 from ACPC exposure treatment showing severe necrosis (>40% cells necrotic). The necrotic cells contain basophilic angulated nuclei with increased cytoplasmic eosinophilia and vacuolation (200X).



Vehicle **200 ACPC 5**

Figure 1


control (n=9)
100 ACPC (n=7)
200 ACPC (n=8)
Mean Neural Damage Score
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1
2
3
4
Par
Oc
PRh
Pir
Co
BM
La
Apir
CA3
CA1

Figure 2

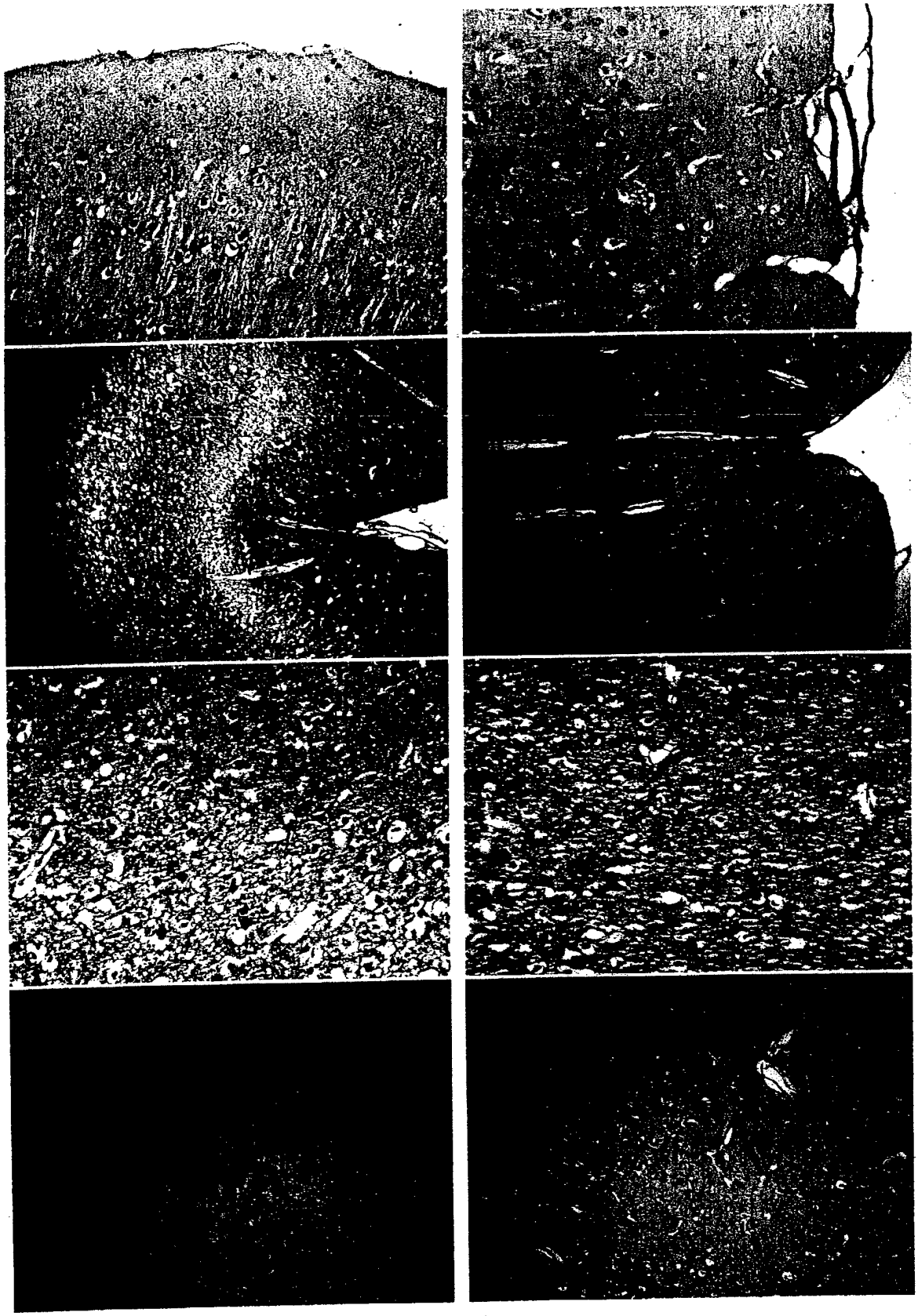

Figure 3

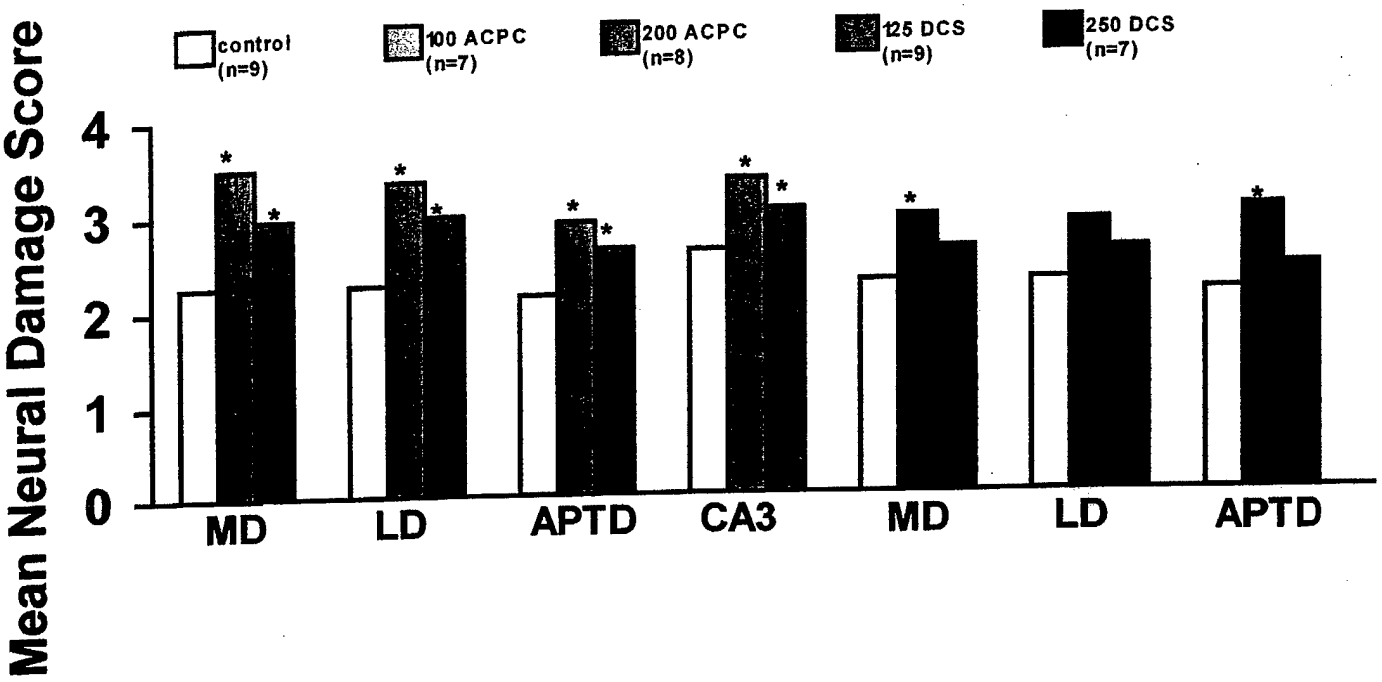
Mean Neural Damage Score
control (n=9)
100 ACPC (n=7)
200 ACPC (n=8)
125 DCS (n=9)
250 DCS (n=7)
0
1
2
3
4
MD
LD
APTD
CA3
MD
LD
APTD

Figure 4

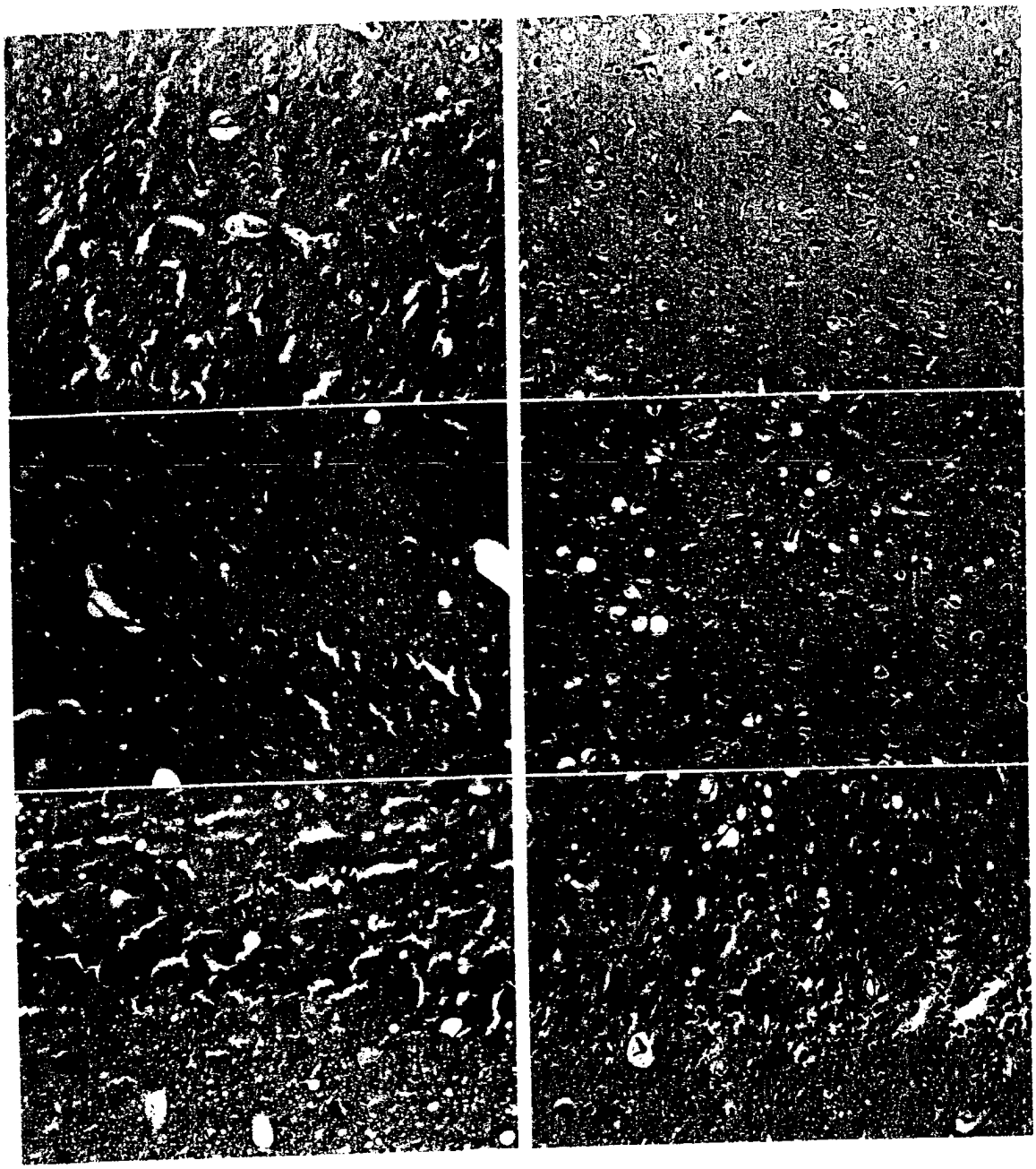

Figure 5